FORM PTO-1390 (REV 11-2000) .US. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		ATTORNEY'S DOCKET NO. 04276.00003 U.S. APPLICATION NO. (Known if Section 371) 09/889325
INTERNATIONAL APPLICATION NO. PCT/JP00/00233	INTERNATIONAL FILING DATE January 19, 2000	PRIORITY DATE CLAIMED January 19, 1999

TITLE OF INVENTION
GENE CAUSATIVE OF ROTHMUND-THOMSON SYNDROME AND GENE PRODUCT

APPLICANT(S) FOR DO/EO/US
Saori KITAO, Akira SHIMAMOTO and Yasuhiro FURUICHI

Applicant herewith submits to the United State Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371 (c)(2).
   a ☒ is attached hereto.
   b ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11-20 below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.
12. ☐ An Assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☒ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4)..
20. ☒ Other items or information: PCT/RO/101 (4 pp.), Copy of WO 00/43522 published July 27, 2000 w/PCT/ISA/210: Specification (41 pp.), Claims 1-14 (3 pp.), Abstract (1 p.), 7 sheets drawings, 60 pp. Sequence Listing; English Translation: Specification (41 pp.), Claims 1-14 (3 pp.), Abstract (1 p.), 7 sheets of drawings, Sequence Listing (60 pp.); PCT/IPEA/416 (1 p.), PCT/IPEA/409 (3 pp.); PCT/RO/105 (1 p.), PCT/ISA/202 (1 p.); PCT/IB/301 (2 pp.); PCT/IPEA/401 (4 pp.); PCT/ISA/220 (3 pp.); PCT/IB/304 (1 p.); PCT/IB/308 (1 p.); PCT/IPEA/402 (1 p.); PCT/IB/332 (1 p.); Letter Pursuant to 37 CFR 1.821(f) w/CRF diskette

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JC18 Rec'd PCT/PTO 13 JUL 2001

U.S. APPLICATION NO. (if known, see 37 CFR 1.5) 09/889325	INTERNATIONAL APPLICATION NO. PCT/JP00/00233	ATTORNEY'S DOCKET NO. 04276.00003

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... $1,000.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... $ 860.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.455(a)(2)) paid to USPTO ..... $ 710.00 International preliminary examination fee (37 CFR 1,482) paid to USPTO) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... $ 690.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) ..... $ 100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$860.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	14 -20 =	0	X $18.00	$	
Independent Claims	6 - 3 =	3	X $ 80.00	$240.00	
Multiple dependent claims (if applicable)			X $270.00	$	
TOTAL OF ABOVE CALCULATIONS =				$1,100.00	
☐Applicant claims small entity status. See 37 CFR 1.27. The fees indicated below above are reduced by 1/2.				$	
SUBTOTAL =				$1,100.00	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f).				$	
TOTAL NATIONAL FEE =				$1,100.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property.				$	
TOTAL FEES ENCLOSED =				$1,100.00	
+				Amount to be: refunded	$
				charged	$

a. ☐ A check in the amount of $_____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 19-0733 in the amount of $1,100.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0733. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Banner & Witcoff, Ltd.
Eleventh Floor
1001 G Street, N.W.
Washington, D.C. 20001-4597
Telephone (202) 508-9100
Date: July 12, 2001

SIGNATURE

Sarah A. Kagan
NAME

32,141
REGISTRATION NUMBER

Attorney Docket No. 04276.00003
International Application No. PCT/JP00/00233

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Saori KITAO, et al.

BOX PCT

National Phase Application
PCT/JP00/00233
Filed: January 19, 2000

Serial No.: Unassigned

Group Art Unit: Unassigned

Filed: CONCURRENTLY HEREWITH

Examiner: Unassigned

For: GENE CAUSATIVE OF ROTHMUND-THOMSON SYNDROME AND GENE PRODUCT

PRELIMINARY AMENDMENT

Assistant Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

Sir:

Preliminarily to the examination of the above-identified application, kindly amend the application as follows:

In the Specification:

Page 1, after the title, insert the following paragraph:

--This is a U.S. National Phase Application Under 35 USC 371 and applicant herewith claims the benefit of priority of PCT/JP00/00233 filed January 19, 2000, which was published under PCT Article 21(2) in Japanese and Application No. JP 11/11218 filed in Japan on January 19, 1999.--

REMARKS

The amendment to the specification is made in accordance with 35 U.S.C. 119, 37 C.F.R. 1.55 and 37 C.F.R. 1.78. No new matter has been added. Entry is requested.

Respectfully submitted,

Sarah A. Kagan
Reg. No. 32,141

July 12, 2001
BANNER & WITCOFF, LTD.
Eleventh Floor
1001 G Street, N.W.
Washington, D.C. 20001-4597
(202) 508-9100

DESCRIPTION

GENE CAUSATIVE OF ROTHMUND-THOMSON SYNDROME AND ITS GENE PRODUCT

Technical Field

The present invention relates to a causative gene of Rothmund-Thomson syndrome, methods for the diagnosis of the disease, and diagnostic agents and therapeutic agents for the disease.

Background Art

Rothmund-Thomson syndrome ((RTS); poikiloderma congenital) is a rare autosomal recessive hereditary disease, the pathophysiology and causative gene of which remain unrevealed. In 1868, a German ophthalmologist, August Rothmund, reported for the first time, 10 patients from an isolated village in Bayern showing crisis of poikiloderma at their youth and exhibiting at a high frequency juvenile cataracts (A. Rothmund, Arch. Ophthalmol. 4:159 (1887)). In 1936, an English ophthalmologist, Sidney Thomson, reported 3 patients with very similar poikiloderma (M.S. Thomson, Br. J. Dermatol. 48:221 (1936)). Two of the three had bone abnormality. Today, these two similar clinical cases are recognized as Rothmund-Thomson syndrome (RTS). Many cases in children of a variety of races affected with this disease have been reported worldwide, and previously over 200 cases of Rothmund-Thomson syndrome had been reported by Vennos et al. (E.M. Vennos et al., J. Am. Acad. Dermatol. 27:750 (1992); E.M. Vennos and W.D. James, Dermatol. Clinics. 13:143 (1995)). Although there is much clinical information on the Rothmund-Thomson syndrome, only clinical background is available for the diagnosis and no method for diagnosis at the laboratory level has been established.

Clinical symptoms of Rothmund-Thomson syndrome include anetoderma and telangiectasia associated with mixed hyperchromic and hypochromic regions during neonatal period, juvenile canities and alopecia prematura, juvenile cataracts, lower stature, congenital skeletal abnormality, and increased risk of mesenchymal tumor. Cytogenetic studies have shown that cells derived from patients with

Rothmund-Thomson syndrome are genetically unstable and often exhibit chromosomal recombination, and acquired somatic cell mosaicism can be found in such cells (K.L. Ying et al., J. Med. Genet. 27:258 (1990); V.M. Der Kaloustian et al., Am. J. Med. Genet. 37:336 (1990); K.H. Orstavik et al., J.Med.Genet. 31:570 (1994); M. Miozzo et al., Int. J. Cancer 77:504 (1998), N.M. Lindor et al., Clin. Genet. 49:124 (1996)). Some of the cytogenetic and clinical findings, including genetic instability in patient cells, juvenile retardation of physical growth, skin abnormality, and high risk of tumorigenesis, are very similar to those findings in Werner syndrome and Bloom syndrome.

Both of the causative genes of Werner syndrome and Bloom syndrome (abbreviated as WRN and BLM, respectively) belong to the RecQ DNA helicase family, and have been identified as homologues of the *E. coli RecQ* gene, which encodes the DNA helicase (K. Nakayama et al., Mol. Gen. Genet. 200:266 (1985)). In addition to WRN and BLM, SGS1 from budding yeast (*S. cerevisiae*) and rqh1$^+$ from fission yeast (*S. pombe*) have been identified as eukaryotic homologues of *E. coli* RecQ DNA helicase. Mutations in the *SGS1* gene are known to result in frequent homologous recombination and non-homologous recombination in budding yeast (*S. cerevisiae*) cells; likewise, rqh1$^+$ mutations are known to result in frequent recombination in S phase in fission yeast (*S. pombe*) (S. Gangloff et al., Mol. Cell. Biol. 14:8391 (1994); P.M. Watt et al., Cell 81:253 (1995); E. Stewart et al., EMBO J. 16:2682 (1997)).

Since a trisomy mosaicism of chromosome 8 was found in two of the three Rothmund-Thomson syndrome patients examined (N.M. Lindor et al., Clin. Genet. 49:124 (1996)), the causative gene of Rothmund-Thomson syndrome has been deduced to be located on chromosome 8. However, the causative gene has not yet been identified.

Disclosure of the Invention

An objective of the present invention is to identify the causative gene of Rothmund-Thomson syndrome. In addition, another objective is to provide methods for the diagnosis of the disease as well as diagnostic and therapeutic agents for the disease.

The inventors had previously isolated a cDNA corresponding to the RecQ4 helicase gene, belonging to the RecQ helicase gene family (Japanese Patent Application No. Hei 9-200387). The inventors considered the possibility that the RecQ4 helicase gene was the causative gene of Rothmund-Thomson syndrome; they therefore isolated the genomic DNA encoding RecQ4 helicase, and evaluated the presence of mutations in the RecQ4 helicase gene from patients with Rothmund-Thomson syndrome by using primers prepared based on the sequence information. The results showed that three of seven Rothmund-Thomson syndrome patients tested contained complexed heterozygotic mutations in the RecQ4 gene. Two of these patients were brothers, and the respective mutant alleles of the two had been inherited from the patients' family members. Aberrant transcription of RecQ4 was specifically found in cells derived from the patients. This suggested that the mutations in the RecQ4 gene result in genetic instability and are the cause of Rothmund-Thomson syndrome. In other words, the inventors have successfully demonstrated for the first time that the RecQ4 gene is the causative gene of Rothmund-Thomson syndrome.

Further, from this fact, they have found that it is possible to diagnose Rothmund-Thomson syndrome by detecting mutations in the RecQ4 helicase gene; moreover, it is possible to treat the disease by compensating for the mutations.

The present invention relates to the causative gene of Rothmund-Thomson syndrome, methods for the diagnosis of the disease, and diagnostic and therapeutic agents for the disease, and more specifically to:

(1) a genomic DNA encoding RecQ4 helicase;

(2) a vector comprising the genomic DNA of (1);

(3) a host cell containing the vector of (2);

(4) a DNA used for diagnosis of Rothmund-Thomson syndrome, which hybridizes to a DNA encoding the RecQ4 helicase or to the expression regulatory region thereof having a chain length of at least 15 nucleotides,

(5) a therapeutic agent for Rothmund-Thomson syndrome, which contains as the effective ingredient a DNA encoding RecQ4 helicase;

(6) a therapeutic agent for Rothmund-Thomson syndrome, which contains as the effective ingredient RecQ4 helicase;

(7) a diagnostic agent for Rothmund-Thomson syndrome, which contains as the effective ingredient an antibody capable of binding to RecQ4 helicase;

(8) a method for the diagnosis of Rothmund-Thomson syndrome, characterized by detecting mutations in the DNA encoding RecQ4 helicase or the expression regulatory region thereof;

(9) a method for the diagnosis of Rothmund-Thomson syndrome of (8), comprising the steps of:

(a) preparing DNA samples from patients;

(b) amplifying the prepared DNA samples by using the DNA of (4) as a primer and determining the base sequence; and

(c) comparing the determined base sequence with that of a healthy, normal person;

(10) a method for the diagnosis of Rothmund-Thomson syndrome of (8), comprising the steps of:

(a) preparing RNA samples from patients;

(b) separating the prepared RNA samples according to their size;

(c) using the DNA of (4) as a probe, hybridizing it to the separated RNAs; and

(d) detecting hybridized RNA and comparing the results with that of a healthy, normal person;

(11) a method for the diagnosis of Rothmund-Thomson syndrome of (8), comprising the steps of:

(a) preparing DNA samples from patients;

(b) amplifying the prepared DNA samples using the DNA of (4) as a primer;

(c) dissociating the amplified DNA into single-stranded DNAs;

(d) fractionating the dissociated single-stranded DNAs on a non-denaturing gel; and

(e) comparing the mobility of the fractionated single-stranded DNAs on the gel with that of the healthy normal control;

(12) a method for the diagnosis of Rothmund-Thomson syndrome of (8), comprising the steps of:

(a) preparing DNA samples from patients;

(b) amplifying the prepared DNA samples using oligonucleotides comprising a base that forms a base pair with the mutated base specific for Rothmund-Thomson syndrome in the DNA encoding RecQ4 helicase, or the expression regulatory region thereof, as at least one of the primers; and

(c) detecting the amplified DNA fragment;

(13) a method for the diagnosis of Rothmund-Thomson syndrome of (8), comprising the steps of: (a) preparing DNA samples from patients;

(b) amplifying the prepared DNA samples using a pair of DNA of (4), which is prepared so as to flank the mutated base specific to Rothmund-Thomson syndrome, as the primer;

(c) hybridizing to the amplified product a pair of oligonucleotides selected from the group of:

(i) an oligonucleotide synthesized such that the base forming a base pair with the mutated base in the amplified product corresponds to the 3'-terminus , and an oligonucleotide synthesized such that the neighboring (on the 3' side) base to said 3'-terminus corresponds to the 5' terminus;

(ii) an oligonucleotide synthesized such that the base forming a base pair with the base of a normal healthy person which corresponds to the mutated base in the amplified product corresponds to the 3'-terminus, and an oligonucleotide synthesized such that a neighboring (on the 3' side) base to said 3'-terminus corresponds to the 5'-terminus;

(iii) an oligonucleotide synthesized such that the base forming a base pair with the mutated base in the amplification product corresponds to the 5'-terminus, and an oligonucleotide synthesized such that the neighboring (on the 5' side) base to said 5'-terminus corresponds to the 3'-terminus;

(iv) an oligonucleotide synthesized such that the base forming a base pair with the base of a normal healthy person which corresponds to the mutated base in the amplified product corresponds to the 5'-terminus, and an oligonucleotide synthesized such that the neighboring (on the 5' side) base to said 5'-terminus corresponds to the 3' terminus

(d) ligating the oligonucleotides; and

(e) detecting the ligated oligonucleotides; and

(14) a method for the diagnosis of Rothmund-Thomson syndrome of (8), comprising the steps of:

(a) preparing protein sample from patients;

(b) contacting an antibody against RecQ4 helicase with the prepared protein sample;

(c) detecting proteins binding to the antibody.

The present invention primarily relates to the causative gene of Rothmund-Thomson syndrome (RTS). The inventors have found that the causative gene of Rothmund-Thomson syndrome encodes human RecQ4 helicase. The base sequences of the genomic DNA encoding RecQ4 helicase determined by the inventors are shown in SEQ ID NO: 1 (expression regulatory region) and SEQ ID NO: 2 (exon and intron regions).

The genomic DNA encoding RecQ4 helicase of the present invention can be obtained by using the entire base sequence described in any of SEQ ID NOs: 1-3, or a part thereof, as a hybridization probe to screen a genomic DNA library. Alternatively the DNA can be amplified and isolated by polymerase chain reaction (PCR) using a genomic DNA or genomic DNA library as the template and using as the primer a part of the base sequence described in SEQ ID NO: 1 or 2.

The genomic DNA of the present invention, as described below, can be used to prepare primers and probes for the diagnosis of Rothmund-Thomson syndrome, to treat Rothmund-Thomson syndrome by gene therapy, and to produce RecQ4 helicase.

The present invention also relates to DNA hybridizing to DNA encoding RecQ4 helicase, or the expression regulatory region thereof, which comprises at least 15 nucleotides and is used for the diagnosis of Rothmund-Thomson syndrome. Preferably, this DNA hybridizes specifically with a DNA encoding RecQ4 helicase or the expression regulatory region thereof.

The term "hybridizing specifically with" herein means that there is no significant cross-hybridization with DNAs or RNAs encoding other proteins under usual hybridization conditions, preferably under

stringent hybridization conditions. Such a DNA doesn't have to be completely complementary to the target sequence but is generally at least 70%, preferably at least 80%, and more preferably at least 90% (for example, 95% or more) identical to the target at the base sequence level.

When used as a primer, the oligonucleotide is generally a 15mer-35mer, preferably a 20mer-28mer.

The primer may be any one of the above, so long as it is capable of amplifying at least a part of the coding region of RecQ4 helicase or a region regulating expression thereof. Such a region includes, for example, the exon region, the intron region, the promoter region and the enhancer region of the RecQ4 helicase gene.

On the other hand, if the oligonucleotide probe is synthetic, it generally consists of at least 15 bases or more. It is possible to use a double-stranded DNA obtained from a clone inserted into a vector, such as plasmid DNA, as a probe, as well as RNA synthesized from the clone by transcription. The region used as a probe can be any region so long as it hybridizes specifically to at least a part of the coding region of RecQ4 helicase or the region regulating expression thereof. Such a region to which the probe hybridizes includes, for example, the exon region, the intron region, the promoter region and the enhancer region of the RecQ4 helicase gene.

Probes such as oligonucleotides, double-stranded DNAs, and RNAs can be used with proper labels. Labeling methods include, for example, end labeling for oligonucleotides, random primer labeling or PCR method for double-stranded DNAs, and in-vitro transcription labeling for RNAs. Compounds useful for labeling include [γ-^{32}P] ATP for end labeling, [α-^{32}P] dCTP or digoxigenin (DIG)-dUTP for random primer labeling and PCR method, and [α-^{32}P] CTP or DIG-UTP for in-vitro transcription labeling.

The "diagnosis of Rothmund-Thomson syndrome", in accordance with the present invention, is characterized by the detection of mutations in the RecQ4 helicase gene. The "diagnosis of Rothmund-Thomson syndrome" in accordance with the present invention includes not only testing of patients exhibiting symptoms of Rothmund-Thomson syndrome due to mutations in the RecQ4 helicase gene,

but also includes testing to judge whether or not the subjects are potentially affected with Rothmund-Thomson syndrome due to the mutations in the RecQ4 helicase gene.

In addition, "detection of mutations in the RecQ4 helicase gene", in accordance with the present invention, includes both detection at the protein level and detection at the DNA and at the RNA levels.

One embodiment of the diagnostic test method, in accordance with the present invention, is a method for directly determining base sequence of the RecQ4 helicase gene from patients. This method comprises the steps of: (a) preparing DNA samples from patients; (b) amplifying prepared DNA samples derived from patients by using the DNA of the present invention as a primer to determine the base sequence; and (c) comparing the determined base sequence with that of a healthy normal person. Direct determination of base sequence includes direct determination of base sequence of RecQ4 genomic DNA and direct determination of base sequence of RecQ4 cDNA.

When the base sequence of genomic DNA of RecQ4 is intended to be determined directly, the genomic DNA is prepared from patients, and the RecQ4 gene is amplified from the genomic DNA from patients by using a sense primer and an antisense primer specific to the RecQ4 gene. It is preferred that the primers are 20mer-28mer in length and that the Tm values thereof are within the range of 65°C-75°C in the amplification of the RecQ4 gene. The RecQ4 genomic DNA, amplified using a sense primer and an antisense primer, is preferably 1 kb-1.5 kb in length. It is preferable to design the sense primer and antisense primer so that the 50 bp-100 bp 5' and 3' ends of the RecQ4 genomic DNA fragment to be amplified overlap with other genomic DNA fragments, thereby covering the entire region of about 6.5 kb of RecQ4 genomic DNA. Further, the expression regulatory region of the RecQ4 gene may be amplified and used as a test subject. The base sequence determination of the amplified fragment can be performed, for example, by the PCR-based method of Hattori et al. (Electrophoresis 13, pp560-565 (1992)). Specifically, the reaction is carried out using a PRISM sequencing kit containing fluorescent dideoxy-terminator (Perkin-Elmer), and using specific primers to the fragment of RecQ4 genomic DNA to be amplified. Subsequently, the base sequence is

determined by an automatic sequencer from Applied Biosystems (Model ABI 373), and the data is analyzed by an attached Macintosh computer. The judgment on the presence of mutations can be formed, for example, by analyzing the base sequence, as a series of peaks of waveforms with four colors by using analytical software for base sequence such as Sequencing Analysis (Applied Biosystems). That is, mutations can be detected by comparing the series of peaks of waveforms representing base sequence of genomic DNA of the normal RecQ4 gene with the series of peaks of waveforms representing base sequence of genomic DNA of a patient's RecQ4 gene. Further, the judgment can be formed through sequence analysis, using base sequence-editing software such as DNASIS. In other words, mutations can be detected by comparing the sequence of normal RecQ4 genomic DNA with the sequence of genomic DNA RecQ4 from patients with a computer.

In the case where the base sequence of RecQ4 cDNA is determined directly, the cDNA is prepared from the RNA sample of patients by reverse transcription, and then the RecQ4 gene is amplified from patients using the sense primer and the antisense primer specific to the RecQ4 gene. It is preferable that the primers are 20 mer-28 mer in length and the Tm values thereof are within the range of 65° C -75° C in the amplification of the RecQ4 gene. The RecQ4 cDNA amplified using the sense primer and antisense primer is preferably 1 kb-1.5 kb in length. It is preferable to design the sense primer and antisense primer such that the 50 bp-100 bp of 5' and 3' ends of the RecQ4 genomic DNA fragment to be amplified overlap with other genomic DNA fragments, and cover the entire region of RecQ4 cDNA which is about 4 kb. The base sequence determination of the amplified fragment can be performed in the same manner as described above for genomic DNA, for example, by the PCR-based method of Hattori et al. (Electrophoresis 13, pp560-565 (1992)). Specifically, the reaction is carried out using a PRISM sequencing kit containing fluorescent dideoxy-terminator (Perkin-Elmer); in which specific primers are used to the fragment of RecQ4 cDNA to be amplified. Subsequently, the base sequence is determined by an automatic sequencer from Applied Biosystems (Model ABI 373), and the data is analyzed by an attached Macintosh computer. The judgment on the presence of mutations can

be formed, for example, by analyzing the base sequence as a series of peaks of waveforms with four colors by using analytical software for base sequence such as Sequencing Analysis (Applied Biosystems). That is, mutations can be detected by comparing the series of peaks of waveforms representing the base sequence of genomic DNA of the normal RecQ4 gene with the series of peaks of waveforms representing the base sequence of genomic DNA of the patients' RecQ4 gene. Further, the judgment can be formed through sequence analysis, using sequence-editing software such as DNASIS. In other words, mutations can be detected by comparing the cDNA sequence of normal RecQ4 with the cDNA sequence of RecQ4 from patients with a computer.

The method for the diagnosis of the present invention includes a variety of other methods in addition to the direct determination method for the base sequence derived from patients as described above. In one embodiment, such a method comprises the steps of: (a) preparing DNA samples from patients; (b) amplifying the prepared DNA samples derived from patients using the DNA of the present invention as a primer; (c) dissociating the amplified DNA into single-stranded DNAs; (d) fractionating the dissociated single-stranded DNAs on a non-denaturing gel; and (e) comparing the mobility of the dissociated single-stranded DNAs on the gel with that of the DNAs from a healthy normal person.

Such a method includes the method of PCR-SSCP (single-strand conformation polymorphism). The PCR-SSCP method is designed based on the principle that two single-stranded DNAs, of which lengths are identical but which base sequences are different, form distinct higher-order structures through their respective intramolecular interactions and therefore show different electrophoretic motilities to each other. That is, the higher-order structure of a single-stranded DNA with a mutation(s) is different from that of a single-stranded DNA without mutation(s), and thus the two exhibit different electrophoretic motilities on a non-denaturing gel. This difference makes it possible to detect the mutation(s) (Orita et al., Proc. Natl. Acad. Sci. USA, 1989, vol. 86, pp2766-pp2770).

The PCR-SSCP method can be used to detect alterations in the sequence of RecQ4 genomic DNA or RecQ4 cDNA. When mutations are

intended to be detected in RecQ4 genomic DNA, the RecQ4 gene is amplified from each of the genomic DNAs of healthy normal person and patient, using a sense primer and an antisense primer specific to the RecQ4 gene. In this experiment, the primers are previously radiolabeled with ^{32}P by an end labeling method. It is preferred that the length of primer is 20 mer-28 mer and the Tm value is within the rage of 65° C -75° C. Further, it is preferable that the RecQ4 genomic DNA to be amplified, using the sense primer and antisense primer, is 300 bp or shorter in length. Preferably, the sense primer and the antisense primer are designed so that the 60 bp-100 bp of 5' and 3' ends of the RecQ4 genomic DNA fragment to be amplified overlap with other genomic DNA fragments, and cover the entire region of RecQ4 genomic DNA, which is about 6.5 kb. The amplified DNA fragment is electrophoresed on a 5% non-denaturing polyacrylamide the thickness and the length of which is 0.3 mm-0.35 mm and 40 cm, respectively. The gel is analyzed by autoradiography and the mobility of the band from the patient is compared with that from a healthy normal person for detection of mutations.

When mutations are intended to be detected in RecQ4 cDNA, the cDNA is prepared from a patient's RNA sample by reverse transcription, and the RecQ4 gene is amplified from cDNAs of healthy normal person and patient using a sense primer and an antisense primer specific to the RecQ4 gene. In this experiment, the primers are previously radiolabeled with ^{32}P by an end labeling method. It is preferred that the length of primer is 20mer-28mer and the Tm value is within the rage of 65° C -75° C. Further, it is preferred that the RecQ4 cDNA to be amplified using the sense primer and antisense primer is 300 bp or shorter in length. Preferably the sense primer and antisense primer are designed so that the 60 bp-100 bp of 5' and 3' ends of the RecQ4 cDNA fragment to be amplified overlap with other cDNA fragments and cover the entire region of RecQ4 cDNA which is about 4 kb. The amplified DNA fragment is electrophoresed on a 5% non-denaturing polyacrylamide the thickness and the length of which is 0.3 mm-0.35 mm and 40 cm, respectively. The gel is analyzes by autoradiography and the mobility of the band from the patient is compared with that from a healthy normal person for detection of

mutations.

The above-described methods for diagnosis are just a few specific examples and those skilled in the art may properly modify the detailed procedures of the methods. In a test of the genomic DNA, the presence of mutations can be tested in the expression regulatory region (promoter region and enhancer region). Moreover, to test if a particular region of genomic DNA or cDNA has a mutation, a DNA fragment containing the site to be tested may be prepared and used for the test instead of a DNA covering the entire region of the RecQ4 gene.

Alternatively, RNA, instead of DNA prepared from patients, can also be used for the detection. Such a method comprises the steps of: (a) preparing RNA samples from patients; (b) separating the prepared RNA samples based on their size; (c) allowing the DNA probe of the present invention, which has been detectably labeled, to hybridize with the separated RNA; and (d) detecting the hybridized RNA and comparing the RNA with that from a healthy normal person. In a specific example, the RNA prepared from patients is electrophoresed, and Northern blotting is performed using the probe DNA of the present invention to detect the presence and intensity of the signal, and/or the difference in mobility on a gel.

In addition to these methods, it is possible to perform the test of the present invention by detecting mutations at positions selected previously.

One embodiment of such a test method comprises the steps of: (a) preparing DNA samples from patients; (b) amplifying the prepared DNA samples from patients using an oligonucleotide containing a base capable of forming a base pair with the mutated base specific for Rothmund-Thomson syndrome in the DNA encoding RecQ4 helicase or the expression regulatory region thereof as at least one of the primers; and (c) detecting the amplified DNA fragment.

Such a method includes, for example, the method of MASA (mutant-allele-specific amplification) (Matsumoto et al., Experimental Medicine 15:2211-2217 (1977); Unexamined Published Japanese Patent Application (JP-A) No. Hei 10-201498).

MASA is a method in which template genomic DNA or cDNA is

amplified by polymerase chain reaction (PCR) using oligonucleotides containing bases capable of forming a base pair with the mutated base as one of the primers, and subsequently subjecting them to gel electrophoresis to detect the mutant alleles.

To conduct this method in accordance with the present invention, a pair of primers (5'-side sense primer and 3'-side antisense primer) is synthesized to amplify the template DNA. Herein, the 5'-side sense primer is synthesized so as to contain a base capable of forming a base pair with the mutated base. The 5'-side sense primer is designed so as to function as a specific primer when a mutation-containing DNA encoding RecQ4 helicase or the expression regulatory region thereof is used as a template, but not when a mutation-free DNA encoding RecQ4 helicase or the expression regulatory region thereof is used. In this case, it is preferred that the base forming a base pair with the mutated base is placed at the 3' end of the 5'-side sense primer. On the other hand, an oligonucleotide primer specifically hybridizing to the region without a mutation is used as the 3'-side sense primer. Polymerase chain reaction is carried out under a condition where the amplification is very efficient, due to the efficient hybridization of the 5'-side sense primer to the template of mutation-containing DNA fragment (abnormal allele), and where the efficiency of amplification is extremely low, due to the incompetence of the 5'-side sense primer in the hybridization to the template of mutation-free DNA fragment (normal allele).

For example, heating once at 95° C for 5 minutes; heating at 94° C for 30 seconds, heating at 50° C for 30 seconds and heating at 72° C for 30 seconds as one cycle, and that for 40 cycles; and a heating at 72° C for 4 minutes are carried out.

Alternatively, polymerase chain reaction can be performed in the same manner, using a 3'-side antisense primer containing a base forming a base pair with the mutated base and a 5'-side sense primer that is an oligonucleotide specifically hybridizing to the region without a mutation.

Thus mutation-containing sample DNA can be amplified efficiently because the DNA can hybridize to the mutation-containing primer. For example, when the amplified DNA is subjected to

electrophoresis, it can be detected as a positive band on the gel. On the other hand, sample DNA from a normal subject is incompetent in the hybridization with a primer containing the mutation and as a result the amplification is not achieved and no band is observed on the gel.

Further, in addition to the detection with the above-mentioned mutation-containing primer, another detection can be carried out using a primer without the mutation (which contains a base incapable of forming a base pair with the mutated base but capable of forming a base pair with the normal base) corresponding to the primer above, to judge whether the subject has the mutation homozygously or heterozygously. That is, when a band is detected with the mutation-containing primer and no band is detected with the mutation-free primer, then the sample DNA can be judged to have the homozygous mutation associated with Rothmund-Thomson syndrome. Alternatively, when a band is observed with both of the two primers, then the sample DNA can be judged to have the mutation heterozygously, or when a band is detected merely with the primer without the mutation, then the DNA can be judged as normal in respect to the tested site.

Another embodiment of the method for diagnosis of the present invention comprises the steps of: (a) preparing DNA samples from patients; (b) amplifying the prepared DNA samples from patients using oligonucleotides prepared as a pair to flank a mutated base specific to Rothmund-Thomson syndrome as a primer; (c) hybridizing to the obtained amplification product any pair of the oligonucleotides of: (i) an oligonucleotide synthesized such that the 3'-terminus thereof corresponds to the base forming a base pair with the mutated base in the amplification product, and an oligonucleotide synthesized such that the neighboring base (on the 3' side) to said 3'-terminus is placed at the 5'-terminus of the synthesized oligonucleotide; (ii) an oligonucleotide synthesized such that the 3'-terminus thereof corresponds to the base forming a base pair with the base from a healthy normal person corresponding to a mutated nucleotide in the amplification product, and an oligonucleotide synthesized such that the neighboring base (on the 3' side) to said 3'-terminus is placed at the 5'-terminus of the synthesized oligonucleotide; (iii) an

oligonucleotide synthesized such that the 5'-terminus thereof corresponds to a base forming a base pair with the mutated base in the amplification product, and an oligonucleotide synthesized such that the neighboring base (on the 5' side) to the 5'-terminus is placed at the 3'-terminus of the synthesized oligonucleotide; (iv) an oligonucleotide synthesized such that the 5'-terminus thereof corresponds to a base forming a base pair with the base from patients corresponding to the mutated base in the amplification product, and an oligonucleotide synthesized such that the neighboring base (on the 5' side) to the 3'-terminus is placed at the 5'-terminus of the synthesized oligonucleotide ; ligating these oligonucleotides; and (d) detecting the ligated oligonucleotides.

Such a detection method includes, for example, OLA (Oligonucleotide Ligation Assay) (Matsumoto et al., Experimental Medicine 15:2211-2217 (1977); JP-A No. Hei 10-201498). First, primers are designed to be placed upstream and downstream of each site to be detected (i.e., sites predicted to contain a mutation) with an appropriate spacing, and then polymerase chain reaction is conducted to amplify genomic DNA fragment or cDNA fragment containing the site to be detected. The distance between each site to be detected and the primer can be selected arbitrarily, but 100 bp-200 bp is preferred. Further, there is no particular limitation on the number of nucleotides in the primer, but a primer of 20mer-30mer is preferred.

On the other hand, based on the base sequence of the RecQ4 helicase gene, an oligonucleotide consisting of 18-30 nucleotides is synthesized so that the above-mentioned site to be detected is placed at the 3' end thereof and that a base forming a base pair with the predicted mutated base is placed at the 3' end (the synthesized oligonucleotide is referred to as "oligonucleotide A"). Further, another oligonucleotide consisting of 18-30 nucleotides is synthesized so that the base neighboring (on the 3' side) to the above-mentioned site to be detected corresponds to the 5' end thereof (the synthesized oligonucleotide is referred to as "oligonucleotide X"). The mutant-type primer can be prepared by mutagenizing the normal sequence using known technique (e.g., by using a mutagenesis kit (In vitro Mutagenesis Kit, TaKaRa Shuzo)), or alternatively

chemically synthesizing the primer based on the sequence designed with a mutation.

According to this preparation, for the convenience of purification and detection of the oligonucleotides ligated through the ligase reaction as described below, it is preferable, for example, to label the 5' end of oligonucleotide A with biotin or the like, to label the 3' end of oligonucleotide X with digoxigenin-11-dideoxy UTP or the like, and to add a phosphate group to the 5' end.

Then, oligonucleotides A and X are annealed with the above-mentioned product of polymerase chain reaction to ligate oligonucleotides A and X with each other. When a mutation of interest is present in the sample DNA, the 3'-end of oligonucleotide A can form a base pair with the mutated base and as a result oligonucleotide A can be connected to oligonucleotide X; and this allows the production of oligonucleotides with labels at both ends (for example, biotin and digoxigenin).

For example, if the product has biotin and digoxigenin at either ends, then the mutation is detected from an arising color reaction, stemming from the absorbance of the product on a plate coated with streptavidin and the subsequent reaction with an anti-digoxigenin antibody conjugated with alkaline phosphatase or the like.

On the contrary, when the sample DNA does not contain the mutation, then the 3'-end of oligonucleotide A cannot form a base pair with the corresponding base in the template DNA, and as a consequence, oligonucleotides A and X cannot be connected with each other.

Accordingly, even when oligonucleotides A and X are labeled, for example, with biotin and digoxigenin, respectively, oligonucleotides with respective labels at respective ends are not formed; and thus even when the ligation reaction product is bound to the plate coated with avidin and the anti-digoxigenin antibody conjugated with alkaline phosphatase or the like is allowed to react thereto, no color reaction is detectable (Delahunty et al., Am. J. Hum. Genet. 58: 1239-1246,1996).

Further, when an oligonucleotide as described below, specifically detecting DNA that doesn't contain mutations at the site

to be tested for detection is used, it is possible to judge whether or not the subject has the mutation homozygously. Specifically, an oligonucleotide containing the normal sequence, that has no mutated nucleotide at the above-mentioned site to be detected (which is referred to as oligonucleotide B), is synthesized in the same manner as oligonucleotide A and then the ligation assay between oligonucleotide B and oligonucleotide X is performed in addition to the ligation assay with oligonucleotide A and oligonucleotide X.

If the experimental result shows a positive color reaction with oligonucleotides A and X but not with oligonucleotides B and X, the sample DNA can be judged to have a homozygous mutation associated with Rothmund-Thomson syndrome. Alternatively, when color development is detected in either assays with oligonucleotides A and X and with oligonucleotide B and X, the DNA can be judged to have a heterozygous mutation; when the color reaction is positive in the assay with oligonucleotides B and X alone, the DNA is judged normal at the tested site.

Alternatively, the mutation can be detected in the same manner as with the above-mentioned oligonucleotides A and X, by the combined use of an oligonucleotide in which a base forming a base pair with the predicted mutated base has been introduced at the 5' end and an oligonucleotide prepared such, so that the base flanking (on the 5' side) to the above-mentioned site to be detected corresponds to the 3' end thereof.

The detection method of the present invention can also be conducted by using antibody capable of binding to RecQ4 helicase. In one embodiment, such a method comprises the steps of: (a) preparing protein sample from patients; (b) contacting antibody against RecQ4 helicase with the prepared protein; and (c) detecting a protein binding to the antibody.

The antibody to be used in the test of the present invention may be a monoclonal antibody or a polyclonal antibody. Antibodies binding to RecQ4 helicase can be prepared by a method known to those skilled in the art (see, for example, Japanese Patent Application No. Hei 9-200387). The antigens utilized to prepare antibodies can be provided, for example, by introducing the gene encoding the antigen

into an appropriate plasmid vector and expressing the gene product in *E. coli* or, alternatively, by introducing the gene into a baculovirus vector and expressing the gene product in insect cells. Alternatively, a synthetic peptide can also be used. The expression vector can be, for example, a vector such as pQE30 (Qiagen) in the case of *E. coli* expression, or a baculovirus vector such as pAcHLT-B (PharMingen). In this case, the purification of the gene product can be simplified by attaching a tag, such as Flag (Chiang, C. et al., EMBO J., 12: 2749-2762 (1993)) or 6xhis (Immunol. Meth. 4: 121-152 (1990)), to the product. The expressed gene product can be purified utilizing the tag.

A number of cases have been discovered where a protein, which has a truncation at the C terminus of the normal RecQ4 helicase, is presumed to be produced by frame shift or a newly generated termination codon due to mutations in the RecQ4 helicase gene in patients with Rothmund-Thomson syndrome (see Examples). Accordingly, it is possible to carry out easily and efficiently the diagnosis of Rothmund-Thomson syndrome by using antibody recognizing the C terminus of RecQ4 helicase (see Japanese Patent Application No. Hei 10-311284).

In addition, when another antibody recognizing the N terminal region of RecQ4 helicase is used in conjunction with the antibody recognizing the C terminal region in the test of the present invention, it is possible to test which of the two, namely an aberrant expression or structural abnormality of the causative gene, is the cause of the disease associated with mutations of the RecQ4 helicase gene in patients. That is, it is believed that when mutations are generated in the causative gene of the disease, caused by the mutation of the RecQ4 helicase, translation products without the normal C terminus are apt to be produced, due to the resulting frame shift and generation of a termination codon. Therefore, mutations are considered to occur frequently in the C terminal region while the N terminal region is normal. Thus, there is a high possibility that the translation product from the causative gene is detectable by antibody against the N terminal region but not by antibody against the C terminus region when there is a structure abnormality in the translation product.

Furthermore, for example, it has been known that, in the WRN helicase gene, the expression level of mRNA corresponding to the gene containing a mutation(s) is markedly reduced (Yamabe, Y. et al., Biochem. Biophys. Res. Commun., 236: 151-154 (1997)). In the RecQ4 helicase gene, the level of mRNA corresponding to the gene was indeed significantly reduced in RTS patients (see Examples). In such cases, it can be expected that the translation product *per se* from the RecQ4 helicase gene containing mutations is sometimes undetectable. In such aberrant expression of the RecQ4 helicase gene (marked reduction of the expression), it is expected that no immunological reaction is detectable by any antibody. Accordingly, the diagnosis of Rothmund-Thomson syndrome can be conducted by the combined use of these two antibodies.

The test, using antibody binding to RecQ4 helicase of the present invention, can be conducted utilizing a variety of publicly known immunological techniques. A preferred method is Western blotting. Specifically, cells from a patient are lysed in a buffer containing detergent, the resulting sample is electrophoresed on an SDS polyacrylamide gel (SDS-PAGE) containing sodium dodecyl sulfate (SDS), the proteins are transferred onto a filter from the gel, the protein of interest can be detected on the filter by using antibody binding to RecQ4 helicase. It is also possible to detect RecQ4 helicase by ELISA (enzyme-linked immunosorbent assay, ELISA; I. Roitt et al., In "Immunology", The C. V. Mosby Co., 1989, pp25.5-25.6) or by immunohistochemical staining on tissue sections. The antibody can be labeled, for example, with an enzyme label such as alkaline phosphatase or horseradish peroxidase. In this case, the protein of interest can be detected through color reaction. In addition, a fluorescent label can be also utilized. The label can also be linked to a secondary antibody recognizing the antibody against the protein of interest for the detection of the protein of interest. Alternatively, the label can also be linked to the antibody against the protein of interest for the detection. By utilizing the above-mentioned method, it is possible to conduct the test for the lack, accumulation or abnormal cellular distribution of RecQ4 helicase.

Thus, the antibody binding to RecQ4 helicase can be used in the diagnosis of Rothmund-Thomson syndrome. When used as a diagnostic agent, the antibody is generally used in a buffer of about pH6-pH8 (for example, phosphate buffer, HEPES buffer, or Tris buffer), and if required, it can be mixed with a carrier (for example, bovine serum albumin of about 1-5% or gelatin of about 0.2%), a preservative (for example, 0.1% sodium azide), and so on.

Samples from patients used in the diagnosis of the present invention can be, if it is a test of genomic DNA, any cells containing genomic DNA derived from patients, and, if it is a test of RNA, cDNA or protein, in principle any cells can be used as far as the cells are derived from the patient and correspond to cells expressing the RecQ4 helicase gene in a healthy normal person. For example, it is possible to use fibroblast cells established from a piece of skin tissue obtained by biopsy, cells prepared by transforming B lymphocytes contained in leukocytes obtained by blood collection by using Epstein-Barr virus, or the like.

The present invention further relates to a therapeutic agent for Rothmund-Thomson syndrome. In one embodiment, such a therapeutic agent comprises a DNA encoding RecQ4 helicase as the effective ingredient. If a DNA encoding RecQ4 helicase is used as the therapeutic agent, full-length genomic DNA encoding RecQ4 helicase or a part thereof, or full-length RecQ4 helicase cDNA (cDNA encoding human RecQ4 helicase is shown in SEQ ID NO: 3) or a part thereof is introduced into an appropriate vector, such as adenoviral vector, retroviral vector, or the like, and then, the resulting DNA is administered intravenously or locally to the diseased site to the patient. The administration method can include an *ex-vivo* method as well as *in-vivo* method.

Thus, the RecQ4 helicase gene containing the mutations can be replaced with the normal gene in the patient, or alternatively the normal gene can be administered to the patient in an additional fashion, thereby treating Rothmund-Thomson syndrome.

In another embodiment associated with the therapeutic agent for Rothmund-Thomson syndrome, RecQ4 helicase is used as an active ingredient. RecQ4 helicase can be prepared as a naturally occurring

protein, or as a recombinant protein provided by genetic recombination techniques. The amino acid sequence of human RecQ4 helicase is shown in SEQ ID NO: 4. The naturally occurring protein can be isolated from tissues or cells highly expressing RecQ4 helicase (for example, thymus and testis, chronic myelogenous leukemia K562 cell, promyelocytic leukemia HL-60cells, HeLa cell) by a method well known to those skilled in the art, for example, affinity chromatography using antibody against RecQ4 helicase. On the other hand, it is possible to prepare the recombinant protein, for example, through culturing cells transformed with DNA encoding RecQ4 helicase (for example, SEQ ID NO: 3). Cells used for the production of the recombinant protein include mammalian cells, insect cells, yeast cells, and *E. coli*. The expression vectors to be used are known to those skilled in the art. Introduction of the vector into host cells and purification of the recombination protein from the resulting transformants can be achieved by using methods known to those skilled in the art. When it is intended to use the obtained RecQ4 helicase as the therapeutic agent for Rothmund-Thomson syndrome, the RecQ4 helicase can be administered directly or alternatively administered after formulating the RecQ4 helicase by a publicly known pharmaceutical production method. For example, the protein can be administered by dissolving the protein into a commonly used pharmaceutical medium, e.g., a neutral solution such as PBS. The dosage depends on various factors, such as the patient's body weight, age, health, and the type of administration method to be used. Those skilled in the art can properly select a suitable dosage. The administration can be performed, for example, subcutaneously, orally, directly to the disease site, etc.

In another embodiment associated with the therapeutic agent for Rothmund-Thomson syndrome, the agent comprises compound capable of stimulating and elevating the expression of RecQ4 helicase as the effective ingredient.

There is the possibility that the onset of Rothmund-Thomson syndrome is closely associated with the reduction of the expression level of the RecQ4 helicase gene. Accordingly, stimulating and elevating the expression of the RecQ4 helicase gene may treat

Rothmund-Thomson syndrome.

A compound capable of stimulating and elevating the expression of the RecQ4 helicase gene can be obtained by inserting the regulatory region (promoter region and enhancer region) responsible for the expression of the RecQ4 helicase gene into a vector containing luciferase as a reporter, introducing the resulting DNA construct into cultured cells, and screening the cells with the introduced DNA for the compound stimulating and elevating the luciferase activity. The base sequence of the expression regulatory region of the human RecQ4 helicase gene is shown in SEQ ID NO: 1. A reporter gene that can be used for this purpose includes the luciferase gene from firefly and the luciferase gene from *Renilla*. Vectors containing these reporter genes include firefly luciferase reporter vector pGL3 and *Renilla* luciferase reporter vector pRL (Promega). The cells to which the DNA is introduced include human 293 cell, HeLa cell, K562 cell and monkey COS7 cell. Using a publicly known method, such as calcium phosphate precipitation method, liposome method, and electroporation method, introduction of the DNA into cells can be performed. When the method is conducted in accordance with the present invention, the reporter gene connected with the promoter region of the human RecQ4 helicase gene is introduced into human or monkey culture cells by the methods as described above and then the cells are cultured. Each of the various types of sample to be tested are added to the culture medium during the culture and then cell extract is prepared 48 hours after the addition of the compound; the luciferase activity in a cell extract is detected by a method as describe in a reference (Yamabe et al., Mol. Cell. Biol., 1998, vol. 18, pp6191-pp6200). Compounds capable of stimulating and elevating the expression of the RecQ4 helicase gene can be identified through the procedures described above. The sample to be tested in the screening includes, for example, cell extract, expression products from gene library, low-molecular-weight synthetic compound, synthetic peptide, natural compound, etc., but is not limited to these examples.

As with the case of the above-mentioned RecQ4 helicase used as a therapeutic agent, when a compound stimulating and elevating the expression of the RecQ4 helicase gene is used as a therapeutic agent

for the disease, it can be administered after formulating the compound by a publicly known pharmaceutical production method.

Brief Description of the Drawings

Figure 1(a) shows a family tree of patients with Rothmund-Thomson syndrome and other members of the family. "I" represents parents; "1" indicates father and "2" indicates mother. Each of half-closed square and circle indicates a genetic carrier with a mutation in one allele of the RecQ4. "II" represents brothers or sisters (1-6) of the patients. Each of completely closed square (II.3, male) and circle (II.6, female) represents a patient with Rothmund-Thomson syndrome who has mutations in both alleles of the RecQ4 gene. II.2, II.4, and II.5 were not patients affected with Rothmund-Thomson syndrome and therefore no analysis was performed for them. The person II.1 indicated by the shaded symbol had been diagnosed as a patient with Rothmund-Thomson syndrome based on the clinical findings.

Figure 1(b) shows the results of analysis for the mutation in the RecQ4 gene in the patients with Rothmund-Thomson syndrome and their parents. Lane I.1 represents the father; lane I.2, the mother; lane II.3, patient II.3; lane II.6, patient II.6. Based on the results, it has been revealed that the mother has a 7-base deletion (mut-1) in one allele of the gene inherited from her parent.

Figure 2 shows the results of direct base sequencing analysis for the RecQ4 gene in its mutational region.

(a) shows base sequences of the region comprising mut-1 (residue 1641-1672 in the protein-coding region) in normal and mutant RecQ4 genes. The region encircled by mut-1 (7-base deletion) was amplified by PCR using genomic DNAs prepared from a healthy normal person and patients II.2 and II.6 with Rothmund-Thomson syndrome, to analyze the base sequences. The results of sequencing of normal and mutant sequences are indicated below.

(b) shows base sequences of the region comprising mut-2 (residue 2257-2280 of the protein-coding region) in normal and mutant RecQ4 genes. The region encircled by mut-2 (point mutation from C to T) was amplified by PCR using genomic DNAs prepared from a healthy

normal person and patients II.2 and II.6 with Rothmund-Thomson syndrome. The sequencing analysis was carried out in the same manner as in (a).

Figure 3 shows a schematic illustration of deleted RecQ4 helicase molecules generated by mut-1 to mut-4. The term "normal" represents the full-length RecQ4 helicase, consisting of the 1208 amino acids deduced from the coding region of the cloned RecQ4 gene. The shaded region represents a helicase domain that is conserved in all RecQ helicases.

Figure 4 shows the investigated results of down-regulated expression of the RecQ4 gene in cells from patients with Rothmund-Thomson syndrome. The transcripts of the RecQ4 gene from cells derived from patients with Rothmund-Thomson syndrome were compared with those from a healthy normal person. Poly(A)$^+$ RNAs from skin fibroblast cells were prepared from patients with Rothmund-Thomson syndrome with mutations in the RecQ4 gene(II.3 and AG05013), from three other patients with Rothmund-Thomson syndrome (AG05139and AG03587A from NIA, Aging Cell Repository; and TC4398 provided by Dr. R. Miller) who had no mutations in the RecQ4 gene, and from a healthy normal person. Northern blot analysis was performed on the RNAs prepared above using a probe prepared from the helicase domain of the RecQ4 gene. The mRNAs were also probed with GAPDH as an internal control. Each lane shows the corresponding results: lane 1, healthy normal person; lane 2, II.3; lane 3, AG05013; lane 4, AG05139; lane 5, AG03587; lane 6, TC4398.

Figure 5 shows a purified partial RecQ4 protein that was synthesized in *E. coli*. 302 amino acids from the C terminal region of RecQ4 were synthesized in *E. coli*. The purified and dialyzed protein was electrophoresed by SDS-PAGE and the gel was CBB-stained. The molecular weight was about 41 kD. Each lane shows the corresponding results: lane M, low molecular weight marker (1 μg); lane 1, purified protein (1 μl); lane 2, purified protein (2 μl).

Figure 6 shows the result of Western blot analysis using normal cells and cells from RTS patients. A 100-μg aliquot of each total cell extract was electrophoresed in a 7.5% polyacrylamide gel and subjected to Western blotting for RecQ4. A control experiment for

total amount of protein was performed with 10-μg aliquots of the respective total cell extracts, which were analyzed by Western blotting for actin. Each lane shows the corresponding results: lane 1, WI38/SV40; lane 2, RTS-B (mut-1 and mut-2); lane 3, RTS-E (mut-3 and mut-4); lane 4, RTS-C (no mutation); lane 5, RTS-F (no mutation).

Figure 7 shows an analysis for intracellular localization of RecQ4 by fluorescent antibody staining. K562 cells were attached onto a glass slide by using a Cytospin and immunostained with anti-RecQ4 antibody of 2 μg/ml (A). The morphology of cells can be recognized in (B), which was observed in the same visual field with transmitted light.

Best Mode for Carrying out the Invention

The present invention is further illustrated in detail below with reference to the Examples, but is not to be construed as being limited thereto.

Example 1. Genomic DNA cloning of the RecQ4 helicase gene

The genomic DNA of the human RecQ4 helicase gene was obtained by screening a P1/PAC library. The P1/PAC library used was obtained from Genome Systems, and the preparation method is described in the Smoller et al. reference (Smoller, et al., Chromosoma, 1991, vol. 100, pp487-pp494). The screening was carried out by PCR using a sense primer, Q4P (5'-CGC TTC TGG AGA AAA TAC CTG CAC-3'/SEQ ID NO: 9), and an antisense primer, Q4Q (5'-TTG GAG CCT CCT CGT TCC CAC ACC-3'/SEQ ID NO: 10), corresponding to the base sequence segments in exon 21 of the RecQ4 gene. The screening was carried out in Genome Systems Co. The isolation and purification of DNA from P1 clone #13447 obtained in the screening was performed by the method as descried in the reference (Smoller, et al., Chromosoma, 1991, vol. 100, pp487-pp494). The genomic base sequence of the RecQ4 gene was determined by using the purified P1 DNA as the template. The determined base sequence of the genomic DNA encoding RecQ4 helicase (exon 1 to exon 21) is shown in SEQ ID NO: 2. The determination of the base sequence was performed by the PCR-based method described by Hattori et al. (Electrophoresis 13, pp560-565 (1992)). That is,

the reaction was conducted by using a PRISM sequencing kit containing fluorescent dideoxy-terminator from Perkin-Elmer. Subsequently, the base sequence information was obtained in an automatic sequencer from Applied Biosystems (Model ABI 373), and then the data was analyzed by an attached Macintosh computer. RecQ4 gene-specific primers used for the base sequence determination are listed in Table1.

Table 1

Q4 137S	(5'-GTT TCC TGA ACG AGC AGT TCG ATC-3'/ SEQ ID NO:11)
Q4 714S	(5'-GCT GCC TCC AGT TGC TTT TGC CTG-3'/ SEQ ID NO:12)
Q4 A2	(5'-TTG GTC GCA GCC CGA TTC AGA TGG-3'/ SEQ ID NO:13)
Q4 A3	(5'-TGG CCC GTG GTA CGC TTC AGA GTG-3'/ SEQ ID NO:14)
Q4 A5	(5'-GAC GGC TGC GCG GGA GAT TCG CTG-3'/ SEQ ID NO:15)
Q4 A9	(5'-CTC AGC CCC TCC AGT CAA GCT AGG-3'/ SEQ ID NO:16)
Q4 C5	(5'-ACC AGT GCC TCA GGT GTC AGC-3'/ SEQ ID NO:17)
Q4 C8	(5'-GGA AAT GTG CTG GGA AAG GAG-3'/ SEQ ID NO:18)
Q4 D5	(5'-ACC AAG AGT CCA CTG CCT ACG-3'/ SEQ ID NO:19)
Q4 D7	(5'-GCT CCG TGG AGT TTG ACA TGG-3'/ SEQ ID NO:20)
Q4 D9	(5'-AGC GCA GCA CCA GGC TCA AGG-3'/ SEQ ID NO:21)
Q4 D13	(5'-GCA CTG CTT CCT GGG CCT CAC AGC-3'/ SEQ ID NO:22)
Q4 E	(5'-GGG TAC AGC GAG CCT TCA TGC AGG-3'/ SEQ ID NO:23)
Q4 E128	(5'-CTC GAT TCC ATT ATC ATT TAC TGC-3'/ SEQ ID NO:24)
Q4 F	(5'-CTG GGC AGG AGC GTG CAG TCA TGC-3'/ SEQ ID NO:25)
Q4 G	(5'-AGG GGA GAG ACG ACC AAC GTG AGG-3'/ SEQ ID NO:26)
Q4 H1	(5'-TTA GGA TCC GGG GTG CTT GTG GAG TTC AGT G-3'/ SEQ ID NO:27)
Q4 H2	(5'-TTA GGA TCC CAG CTT ACC GTA CAG GCT TTG G-3'/ SEQ ID NO:28)
Q4 K	(5'-TCC TGG CTG TGA AGA GGC TGG TAC-3'/ SEQ ID NO:29)
Q4 L	(5'-ATC CCC CAA TGC AGT GCA GTC AGC-3'/ SEQ ID NO:30)
Q4 U	(5'-AAT CTG GGA CCT CAC TGT GAC ATC-3'/ SEQ ID NO:31)
Q4 Z	(5'-AGG GTG CCT TTC AGA TTG GCC TTG-3'/ SEQ ID NO:32)

The base sequence analysis revealed that the RecQ4 gene consists of 21 exons and 20 introns, and its full length is about 6.5 kb.

Example 2. Cloning of the promoter region of the RecQ4 helicase gene

DNA from P1 clone #13447, containing the genomic DNA of the human RecQ4 helicase gene, was digested with restriction enzymes BamHI and BglII (TaKaRa Shuzo), and the plasmid vector pBluescriptII KS+ was

digested with BamHI. The resulting digested DNAs were mixed with each other and then T4 DNA ligase (TaKaRa Shuzo) was added thereto for ligation reaction. *E. coli* competent cells, DH5α (Toyobo), were transformed with the reaction product and the resulting *E. coli* colonies were screened by PCR to determine whether or not the DNA from each colony contained a 5' upstream region of human genomic DNA of RecQ4. The screening for clones containing the 5' upstream region was carried out by using a sense primer, Q4 S (5'-TCA CAA CTT CTG ATC CCT GGT GAG-3'/SEQ ID NO: 5), and an antisense primer, Q4 R (5'-GAG GGT CTT CCT CAA CTG CTA CAG-3'/SEQ ID NO: 6), for amplifying a 247-bp segment of genomic DNA of RecQ4 sequence (residue 1399 to residue 1645). The bacteria were transferred from the colony into a PCR reaction solution using a toothpick. The following PCR experiment was conducted: denaturation at 95°C for 5 minutes; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds; and final extension reaction at 72°C for 5 minutes. After the reaction was completed, the PCR solution was analyzed by electrophoresis on a 2% agarose gel. The colony, in which a 247-bp band was detected, was judged to be positive. The bacteria derived from each of the resulting positive colonies were cultured in 3-ml LB medium. The alkali-SDS method was used to prepare plasmid DNA. Then the base sequence of the upstream region of the genomic DNA of RecQ4 was determined by using the plasmid DNA as a template and using the following primers: Q4 A14 (5'-CAA TGG GAG GCG TCA ACG TCA TCG-3'/SEQ ID NO: 7) and Q4 A15 (5'-GAG GCG AAA GAG CGG AGG GTC CAG-3'/SEQ ID NO: 8). The transcription initiation site of the RecQ4 gene was previously determined by cap-site PCR (Kitao, S. et al., Genomics, 1998, vol. 54, pp443-pp452; Japanese Patent Application No. Hei 9-200387). The cap-site PCR is a method for accurately determining the initial base in transcription. The transcription initiation site of the human WRN gene has also been determined by this method (Yamabe et al., Mol. Cell. Biol., 1998, vol. 18, pp6191-pp6200). The determined transcription initiation site corresponds to the first residue (residue 1) in the base sequence of genomic DNA of RecQ4 as well as in the base sequence of RecQ4 cDNA. The base sequence of upstream region of the RecQ4 gene was analyzed

using the obtained genomic DNA. The analysis revealed a 5' upstream sequence of 679 bp (SEQ ID NO: 1) from the transcription initiation base.

Example 3. Detection of mutations in the RecQ4 helicase gene in patients with Rothmund-Thomson syndrome

The inventors had previously cloned and analyzed two novel human helicase genes, RecQ4 and RecQ5, belonging to the RecQ helicase gene family (see Japanese Patent Application No. Hei 9-200387; Japanese Patent Application No. Hei 10-81492; and Kitao, S. et al., Genomics, 1998, vol. 54, pp443-pp452). Together, with these two novel genes, there are 5 members belonging to the human RecQ helicase gene family, including RecQ1 (M. Seki et al., Nucleic Acids Res. 22:4566 (1994); K.L. Puranam et al., J. Biol. Chem. 269:29838 (1994)), BLM (N.A. Ellis et al., Cell 83:655 (1995)), WRN (C.-E. Yu et al., Science 272:258 (1996)), RecQ4 and RecQ5.

Northern blot analysis for these five RecQ helicase genes revealed that, RecQ5, like RecQ1, was observed to be ubiquitously expressed through all the tissues and organs, while markedly high level expression was observed in the thymus and testis and high levels in the pancreas, small intestine and large intestine in a tissue-specific manner, like BLM and WRN for RecQ4. The fact that BLM and WRN are causative genes of Bloom syndrome and Werner syndrome, respectively, gave the thought that the RecQ4 gene was also involved in some diseases. The present inventors focused on Rothmund-Thomson syndrome, which exhibits similar symptoms to those of Bloom syndrome and Werner syndrome but for which the causative gene has not yet been identified. The inventors analyzed mutations in the RecQ4 gene using cells and DNA derived from two patients, (brothers) II.3 and II.6, who have previously been identified and reported as patients with Rothmund-Thomson syndrome by Lindor et al. (N.M. Lindor et al., Clin. Genet. 49:124 (1996)) , cells and DNA from their parents and cells and DNA from patients with Rothmund-Thomson syndrome unrelated to the above-mentioned patients.

Specifically, full-length open reading frame of RecQ4 cDNA, as well as all exons of the RecQ4 gene, were first amplified by PCR from

the two RTS patients, II.3 and II.6, and their parents reported by Lindor et al. to determine and compare the base sequences.

In order to amplify the full-length open reading frame of RecQ4 cDNA, total RNA was extracted from fibroblast cell lines derived from the two RTS patients by AGPC method (Chomczynski et al., Analytical Biochemistry, 1987, vol.162, pp.156-159), the mRNA was prepared from the total RNA by using Oligo(dT)30 cellulose beads, and subsequently, cDNA was synthesized through the reverse transcription (RT) reaction. PCR for amplifying the full-length open reading frame of RecQ4 cDNA was conducted as follows (Table 2):

Table 2

Composition of primary reaction solution:		
template DNA	1	μl
20 μM each primer (A5/A7)	0.5	μl X 2
10 X buffer (Clontech)	2.5	μl
2.5 mM dNTPs	2	μl
DMSO	1.25	μl
Klen Taq. polymerase (Clontech)	0.5	μl
dH_2O	16.75	μl
(total volume 25 μl)		

Composition of secondary reaction solution:		
template DNA	1	μl
20 μM each primer (A6/A8)	0.5	μl X 2
10 X buffer (Clontech)	2.5	μl
2.5 mM dNTPs	2	μl
DMSO	1.25	μl
Klen Taq. polymerase (Clontech)	0.5	μl
dH_2O	16.75	μl
(total volume 25 μl)		

Reaction condition : 1 X (94°C 1 min)
5 X (94°C 30 sec, 72°C 4 min)
5 X (94°C 30 sec, 72°C 4 min)
25 X (94°C 30 sec, 68°C 4 min)
1 X (4°C ∞)

Primer sequence

A5 5'-GAC GGC TGC GCG GGA GAT TCG CTG-3'/ SEQ ID No. 15
A6 5'-AGA TTC GCT GGA CGA TCG CAA GCG-3'/ SEQ ID No. 33
A7 5'-CAG GTT TTG CCC AGG TCC TCA GTC-3'/ SEQ ID No. 34
A5 5'-GTC ACT GGC CTA GCC TCT GAC AAC-3'/ SEQ ID No. 35

The resulting PCR product was excised from an agarose gel and

purified. Then, the product was subcloned into a pCR2.1 vector (Invitrogen). The determination of the base sequence was performed by the PCR-based method described by Hattori et al. (Electrophoresis 13, pp560-565 (1992)). That is, the sequencing reaction was carried out using PRISM sequencing kit containing fluorescent dideoxy-terminator from Perkin-Elmer. Primers used for the determination of base sequence were as follows (Table 3):

Table 3

Q4 A2	(5'-TTG GTC GCA GCC CGA TTC AGA TGG-3'/ SEQ ID NO:13)
Q4 U	(5'-AAT CTG GGA CCT CAC TGT GAC ATC-3'/ SEQ ID NO:31)
Q4 T	(5'-TCA TCT AAG GCA TCC ACC CCA AAG-3'/ SEQ ID NO:36)
Q4 S	(5'-TCA CAA CTT CTG ATC CCT GGT GAG-3'/ SEQ ID NO:5)
Q4 A9	(5'-CTC AGC CCC TCC AGT CAA GCT AGG-3'/ SEQ ID NO:16)
Q4 137S	(5'-GTT TCC TGA ACG AGC AGT TCG ATC-3'/ SEQ ID NO:11)
Q4 F	(5'-CTG GGC AGG AGC GTG CAG TCA TGC-3'/ SEQ ID NO:25)
Q4 714S	(5'-GCT GCC TCC AGT TGC TTT TGC CTG-3'/ SEQ ID NO:12)
Q4 975S	(5'-GGA CAC AGA CCA GGC ACT GTT GAC-3'/ SEQ ID NO:38)
Q4 E	(5'-GGG TAC AGC GAG CCT TCA TGC AGG-3'/ SEQ ID NO:23)
Q4 K	(5'-TCC TGG CTG TGA AGA GGC TGG TAC-3'/ SEQ ID NO:29)
Q4 H2	(5'-TTA GGA TCC CAG CTT ACC GTA CAG GCT TTG G-3'/ SEQ ID NO:28)
Q4 H1	(5'-TTA GGA TCC GGG GTG CTT GTG GAG TTC AGT G-3'/ SEQ ID NO:27)
Q4 2314S	(5'-CAG GCC AGA CTC CAG GAT TGG GAG-3'/ SEQ ID NO:39)

Subsequently, the base sequence information was obtained in an automatic sequencer from Applied Biosystems (Model ABI 373), and the data was analyzed by an attached Macintosh computer. The obtained base sequences of the full-length open reading frames from two RTS patients were compared with previously reported base sequence of RecQ4 cDNA (Japanese Patent Application No. Hei 9-200387) using base sequence editing software, DNASIS.

Subsequently, in order to amplify exons of the RecQ4 gene from genomic DNAs, cultured fibroblast cells, which were obtained from the two RTS patients, II.3 and II.6, and their parents, were washed with PBS, and then suspended in TNE buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA). Then, an equal volume of TNE buffer, containing 2% SDS and 200 μg/ml Proteinase K, was added to each suspension and the resulting cell suspension was mixed by frequently turning it upside down at room temperature for 1 hour. The mixture was incubated at 42° C overnight and then DNA was extracted from the

mixture. The extracted DNA was treated 3 times with an equal volume of phenol to remove proteins. Subsequently, the sample was ethanol-precipitated to give purified genomic DNA. By PCR using each genomic DNA as a template, the region containing exons 9, 10 and 11 of the RecQ4 gene was amplified using a sense primer, Q4 C8 (5'-GGA AAT GTG CTG GGA AAG GAG-3'/SEQ ID NO: 18), and an antisense primer, Q4 C5 (5'-ACC AGT GCC TCA GGT GTC AGC-3'/SEQ ID NO: 17); likewise, the region containing exons 13, 14 and 15 of the RecQ4 gene was amplified using a sense primer, Q4 E128 (5'-CTC GAT TCC ATT ATC ATT TAC TGC-3'/SEQ ID NO: 24), and an antisense primer, Q4 D1 (5'-CTC TTC ACA GCC AGG AAG TCC-3'/SEQ ID NO: 40). The following PCR reaction was conducted: denaturation at 95° C for 5 minutes; 35 cycles of denaturation at 94° C for 30 seconds, annealing at 60° C for 30 seconds and extension at 72° C for 60 seconds; and the final reaction at 72° C for 5 minutes. The amplified DNA fragments were purified, and, using these DNAs as templates, the base sequence of the region containing exons 9, 10 and 11 in the RecQ4 gene was determined with Q4 C8 primer and the base sequence of the region containing exons 13, 14 and 15 in the RecQ4 gene was determined with Q4 D3 primer (5'-AGA GCT GGT GTC CCC GTG GAC-3'/SEQ ID NO: 41). The determination of base sequences was performed using a PCR-based method described by Hattori et al. (Electrophoresis 13, pp560-565 (1992)). That is, the sequencing reaction was conducted by using a PRISM sequencing kit containing fluorescent dideoxy-terminator from Perkin-Elmer. Subsequently, the base sequence information was obtained in an automatic sequencer from Applied Biosystems (Model ABI 373), and then the data was analyzed by an attached Macintosh computer. The obtained base sequences from patients and their parents were compared to each other by using the base sequence editing software, DNASIS.

Based on the above-described base sequence analysis of the RecQ4 helicase gene, as described below, it has been clarified that both of the patients with Rothmund-Thomson syndrome in this family have heterozygous mutations. The family tree of the patients with Rothmund-Thomson syndrome is shown in Figure 1(a), and the result of mutation analysis of in this family is shown in Figure 1(b) and Figure 2.

One mutation (referred to as mut-1) is present in exon 10, and it is a 7-base deletion (namely, GGCCTGC of position 1650-1656 in the base sequence of protein coding region (which corresponds to nucleotide 1734-1740 in SEQ ID NO: 3) (See Figure 2(a)). This deletion causes frame shit, and as a result a termination codon TGA is generated 14-bases downstream of the deletion. Primers Q4 C1 (5'-TCT GGC CTG CCA CCG TGT CTC-3'/SEQ ID NO: 42) and Q4 C3 (5'-TGG TCA TGC CCG AGT GTA TGC-3'/SEQ ID NO: 43) were designed so that the mutation site of mut-1 was located between the two primers. The residue 1624-1675 in the protein coding region of the RecQ4 gene (position 1708-1759 in SEQ ID NO: 3) (52 bp) in each of DNAs from the parents (I.1 and I.2) and from the patients (II.3 and II.6) was amplified by PCR using these primers. The resulting DNA fragment was fractionated by electrophoretic separation in a 15% polyacrylamide gel to analyze the mutations. Thus, the presence of the mut-1 mutation was detected based on the difference in electrophoretic mobility (Figure 1(b)). The analytical result showed that mut-1 was derived from the mother.

The other mutation (referred to as mut-2) is a point mutation from C to T at residue 2269 in the protein-coding region (position 2353 in SEQ ID NO: 3). The original codon CAG (Gln) has been converted to be a termination codon TAG (Figure 2(b)). Both of mut-1 and mut-2 have mutations in the helicase domain of RecQ4 helicase, and it is presumed that the translation of transcripts for these defective genes is prematurely terminated, and these produce markedly smaller proteins (60 kDa and 82 kDa, respectively), as compared with the molecular weight of 133 kDa expected from the full length of the coding region for RecQ4 helicase. The results obtained by the mutation analysis are summarized in Table 4. The deduced truncated protein products are shown in Figure 3. The same sequencing analysis was carried out by using DNAs prepared from other subjects belonging to this family. In this analysis, mut-1 was detected in patients II.3 and II.6 with Rothmund-Thomson syndrome as well as in I.2 cells derived from their mother; mut-2 was detected in patients II.3 and II.6 with Rothmund-Thomson syndrome as well as in I.1 cells derived from their father. That is, it was verified that mut-1 and mut-2 were derived from the mother and father, respectively, and the respective mutations

had been inherited from the phenotypically healthy parents having single mutations.

In addition to these mutations specifically related to this family, another heterozygotic mutation has been found in the cell line derived from a patient with Rothmund-Thomson syndrome unrelated to the above-mentioned family. The cell line (No. AG05013) has been deposited in "Aging Cell Repository" of "National Institute of Aging (NIA)" in the USA. The mutation in the cell line were detected by amplifying the full-length open reading frame of RecQ4 cDNA and all exon regions of the RecQ4 gene by PCR, determining the base sequences thereof and comparing them with the normal sequence. Procedures for amplification of the full-length open reading frame of RecQ4 cDNA, subcloning and base sequence determination are as described above. In order to amplify exons of the RecQ4 gene from this patient, genomic DNA was prepared from fibroblast cells of the patient by the same method described above. By using the genomic DNA as a template, the region containing exons 14 and 15 of the RecQ4 gene was amplified by PCR using sense primer, Q4 D3 (5'-AGA GCT GGT GTC CCC GTG GAC-3'/SEQ ID NO: 41), and antisense primer, Q4 D2 (5'-TGG GAA CAC GCG CTG TAC CAG-3'/SEQ ID NO: 44). The region containing exons 12 and 13 of the RecQ4 gene was also amplified by PCR with sense primer, Q4 D11 (5'-GCC TCA CAC CAC TGC CGC CTC TGG-3'/SEQ ID NO: 45), and antisense primer, Q4 D12 (5'-GAC AGG CAG ATG GTC AGT GGG ATG-3'/SEQ ID NO: 46). The condition for PCR was as described above. The amplified DNA fragments were purified, and using the DNAs as templates, the base sequence of the region containing exons 14 and 15 in the RecQ4 gene was determined with Q4 D2 primer as well as the base sequence of the region containing exons 12 and 13 of the RecQ4 gene was determined with Q4 D11 primer. The results show that one of these mutations was a 2-base deletion (mut-3) and the other was a point mutation from G to T at the boundary between intron 12 and exon 13, which destroys the splicing donor consensus sequence (mut-4). It has been revealed that both mutations might cause frame shift for the translation downstream of the helicase domain, which respectively generate truncated protein products of 881 amino acids and 794 amino acids (Table 4 and Figure 3).

Table 4

Rec Q4 gene mutations shown in RTS patients cell				
variant	mutation	exon	situation	deriviation
conjugated heterozygote	1650 7 bases deletion (mut-1)	10	frameshift	Mexican-American
	C2269 (mut-2)	14	nonsense mutation	
conjugated heterozygote	2492 2 bases deletion (mut-1)	15	frameshift	white
	C2269 (mut-2)	13	frameshift	

In order to clarify whether or not the patients with Rothmund-Thomson syndrome carrying the mutations, mut-1 and mut-2, also have mutations in the WRN helicase gene or BLM helicase gene, poly(A)$^+$RNAs from II.3 cells and AG05013 cells were reverse transcribed into cDNAs and base sequences were analyzed by amplifying the full-length open reading frames of the cDNAs by PCR, using the cDNAs as templates. The amplified region of WRN cDNA corresponded to the residues 188-4555 of GenBank accession No. L76937 and the region of BLM cDNA corresponded to the residues 57-4370 of GenBank accession No. U39817. However, no mutations were found in the WRN gene and BLM gene, which suggested that the WRN gene and BLM gene were not involved in Rothmund-Thomson syndrome. Based on the results described above, it can be concluded that mutations in the RecQ4 gene are associated with Rothmund-Thomson syndrome. Furthermore, the results suggest that neither normal WRN helicase nor normal BLM helicase can rescue the deficiency caused by the mutations in the RecQ4 gene in patients with Rothmund-Thomson syndrome.

As described above, the inventors performed mutational analysis for DNAs from 7 patients who had been clinically diagnosed as affected with Rothmund-Thomson syndrome, and found mutations in the RecQ4 gene in 3 patients including II.3 and II.6 belonging to the same family.

Example 4. Northern blot analysis of the cells from patients with Rothmund-Thomson syndrome

To evaluate the relationship between mutations in the RecQ4 gene and pathogenesis of Rothmund-Thomson syndrome from a different viewpoint, RecQ4 mRNA from cells derived from 5 patients with Rothmund-Thomson syndrome were compared with that from a healthy normal person by Northern blot analysis (Figure 4). Total RNA was first extracted from fibroblast cells from patients by AGPC method (Chomczynski et al., Analytical Biochemistry, 1987, vol. 162, pp156-pp159), and poly(A)$^+$ RNA was purified from the resulting total RNA by using oligo(dT) latex beads. The poly(A)$^+$ RNA (5 μg) was electrophoresed on a 1%agarose gel and then denatured with an alkaline solution. Then, the RNA was transferred onto a nylon filter. The 321-bp fragment consisting of residue 2013-2333 in the RecQ4 cDNA (GenBank accession No. AB006532) was amplified by PCR and then purified. The resulting fragment was radiolabeled with [α-^{32}P] dCTP by using a Random Primer DNA Labeling Kit Ver.2 (TaKaRa Shuzo, code no. 6045) and used as a probe. The filter was incubated in a solution containing 5X SSPE buffer, 50% formamide, 2% sodium dodecyl sulfate (SDS), 10X Denhardt's solution, 100 μg/ml salmon sperm DNA, and 1 X 10^7 cpm/ml [α-^{32}P]dCTP-labeled probe DNA at 42°C overnight. Subsequently, the filter was washed 3 times with 2X SSC-0.1% SDS at room temperature and then washed with 0.2X SSC-0.1% SDS at 65°C for 30 minutes. The radioactivity was detected by autography with a BAS1500 system (Fuji film).

The results show that the level of RecQ4 mRNA of about 4 kb was significantly reduced in fibroblast cells derived from II.3 (lane 2), as compared with that in fibroblast cells from healthy normal person (lane 1). Such specific reduction in the level of defective mRNA has also been observed in the expression of WRN gene in fibroblast cells derived from Werner patients and B lymphoblast-like cells transformed with Epstein-Barr virus (Y. Yamabe et al., Biochem. Biophys. Res. Commun. 236:151 (1997)). There are a number of reports indicating that nonsense codons influence RNA metabolism in vertebrate cells, that specific turnover of defective mRNA is

stimulated and, as a result, similar downregulation of the expression can be found in other genetic diseases (L.E. Maquat, RNA 1:456 (1995); L.E. Maquat, Am. J. Hum. Genet. 59:279 (1996)). On the other hand, two types of mRNAs with normal and shorter sizes were detected in Northern blot analysis of mRNA prepared from the other patient(AG05013), carrying the heterozygotic mutations of the 2-base deletion and the point mutation at the 3'-splice site (Figure 4, lane 3). The short mRNA is presumed to be the product of aberrant selective splicing, due to the mutation at the splice donor site, and is presumed to be the major molecular species for RecQ4 mRNA in this sample. On the other hand, transcripts of the RecQ4 gene, which were derived from three (lanes 4-6) of the remaining four patients with Rothmund-Thomson syndrome in whom no mutations had been found in the RecQ4 gene, were essentially the same as that from normal person (lane 1). These result, with respect to the transcript of the RecQ4 gene, is consistent with results obtained in the mutation analysis of DNA sequence. Thus, it was verified that mutations in the RecQ4 gene resulted in the disease in the patients with Rothmund-Thomson syndrome, II.3, II.6, and AG05013.

Diagnosis for Rothmund-Thomson syndrome on patients who are suspected to carry this disease have been previously based on relatively broad clinical findings and, thus, has been less accurate and less reliable. It is suggested that there may exist mutations in other genes (or other gene families) in the patients, in whom no mutations had been found in the RecQ4 gene, of the 7 patients with Rothmund-Thomson syndrome, or, alternatively, the diagnosis of Rothmund-Thomson syndrome may be wrong and in actuality the patient may be afflicted with another disease, one that exhibits similar clinical manifestations. In addition, there is a possibility that the clinical symptoms utilized as an index for the diagnosis of Rothmund-Thomson syndrome are too broad. The disease name "Rothmund-Thomson syndrome" is often used widely for patients exhibiting similar but ambiguous symptoms (E.M. Vennos et al., J. Am. Acad. Dermatol. 27:750 (1992); E.M. Vennos and W.D. James, Dermatol. Clinics. 13:143 (1995)). The diagnosis of Rothmund-Thomson syndrome can be made more accurately by utilizing

gene diagnosis with the RecQ4 gene sequence.

Example 5. Preparation of anti-RecQ4 helicase monoclonal antibody

A DNA fragment containing the nucleotides 2803 to 3711 of SEQ ID NO: 3, encoding the C terminal region of RecQ4, was inserted downstream to lac promoter/operator in an *E. coli* expression vector, pQE30 plasmid (QIAGEN). The plasmid DNA was transformed into an E. coli M15 strain containing a plasmid encoding lac repressor. The resulting transformant was cultured in LB medium (1% Bacto tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.0)containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. Once the bacterial turbidity reached O.D.600=0.6-0.7, then 1 mM IPTG(isopropyl-β-D-thiogalactopyranoside)was added to the culture to induce expression.

The *E. coli* was harvested by centrifugation, and then lysed by sonication in Buffer A (50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 0.1 mM DTT, 5% glycerol, 1 mM PMSF) containing 2% NP-40. Then, centrifugal separation was repeated twice to obtain an insoluble precipitate. The resulting precipitated fraction was suspended in Buffer A, and mixed well with an equal volume of 1 M sucrose and 2 volumes of Percoll (SIGMA; colloidal PVP coated silica for cell separation). The mixture was treated by ultracentrifugation (Beckman ultracentrifuge L7-65, SW28 rotor, 20000 rpm, 15° C, 30 min) to yield protein inclusion body in the lowest layer. The resulting sample was washed 4 times with 50 mM Tris-HCl (pH 8.0) and then dissolved in Buffer G (6 M guanidine-HCl, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8.0). The Buffer G was replaced with Buffer B (8 M-1 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8.0) by dialysis, and then further replaced with PBS. After the dialysis, the sample was concentrated by centrifugation in a conventional centrifuge concentrator/desalting device CENTRIPLUS 10 (Amicon). The above procedures provided a C terminal region (residue 2803-3711 in SEQ ID NO: 3; residue 907-120 in SEQ ID NO: 4 (amino acid sequence)) recombinant protein of RecQ4 helicase (Figure 5).

The purified recombinant protein (50 μg), mixed with Freund's complete adjuvant, was intraperitoneally given to BALB/c mice (7-week old, female) as primary immunization. 23 days after the primary immunization, the secondary immunization was carried out by

intraperitoneal administration of the purified recombinant protein (50 μg) mixed with Freund's incomplete adjuvant. 30 days after the secondary immunization, the final immunization was performed by intravenous administration of the purified recombinant protein (25 μg). 3 days after, spleens was excised from the mice. The separated spleen cells and cells of NS-1 line were fused with each other in the presence of polyethylene glycol and suspended in HAT selection medium. 100-μl aliquots of the cell suspension were placed in wells of 96-well plates (560 wells in total) to cultivate the cells. In order to evaluate the antibody production in hybridomas, primary screening was performed by testing each culture supernatant in the 560 wells according to the antigen-solid-phase ELISA method using the purified recombinant RecQ4 helicase protein as the antigen. The result showed that 450 wells were positive. Among the wells, 55 wells that exhibited high values measured by ELISA were selected, and the corresponding cells were further cultured. The secondary screening was carried out in the same manner as in the primary screening according to the antigen-solid-phase ELISA method. All the 55 wells selected were evaluated as positive. The top fourteen wells in the measured values by ELISA were selected and the corresponding cells were treated by limiting dilution method to clone the hybridomas of interest. Hybridoma clones that were evaluated positive in ELISA were established as monoclonal antibody-producing clones. The established hybridomas were inoculated into BALB/c mice to prepare ascites, and purification of the antibody from the ascites was performed by the ammonium sulfate salting method. A clone K6314 was selected from the resulting 14 clones and the monoclonal antibody produced by this clone was further used as anti-RecQ4 helicase antibody in the experiments described below.

Example 6. Western blot analysis of cells from patients with Rothmund-Thomson syndrome

Western blot analysis for RecQ4 protein was carried out using human normal cells and cells from patients with Rothmund-Thomson syndrome. Primary cultured fibroblast cells, which had been isolated from a healthy normal person as well as from patients, were transformed

with SV40 large T antigen to prepare strains of culture cells. These cultured cells were washed with PBS and then suspended in TNE (40 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA). The cells were harvested by centrifugation and then suspended in Lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% NP-40, 1 mM PMSF). The suspension was mixed by frequently turning it upside down at 4° C for 30 minutes. After centrifugal separation, the resulting supernatant was obtained as the total cell extract. The concentration of protein was measured by using a Protein Assay DyeReagent Concentrate (BIO-RAD).

The prepared total cell extract was subjected to SDS-PAGE (SDS-polyacrylamide gel electrophoresis) according to the method of Laemmli (Laemmli (1970) Nature, vol. 227, p680-685)). Proteins were fractionated on a gel by electrophoresis and then were electrophoretically transferred from the gel onto a nitrocellulose filter (Imobilon transfer membrane; MILLIPORE) in a transfer buffer (20% methanol, 4.8 mM Tris, 3.9 mM glycine, 3.75% SDS) by using a TRANS-BLOT SD (BIO-RAD) at 20 V at room temperature for 1 hour. Blocking of this filter was carried out in PBS containing 5% skimmed milk. The filter was incubated with the primary antibody at room temperature for 2 hours and then washed with 0.05% Tween 20/PBS solution (PBS-T). Subsequently, the filter was incubated with the secondary antibody at room temperature for 1 hour and then washed with PBS-T. Then signal detection was carried out by using ECL Western blotting detection reagents (Amersham).

The primary antibodies used were 2 μg/ml anti-RecQ4 helicase mouse monoclonal antibody K6314 and 0.2 μg/ml anti-actin goat polyclonal antibody sc-1616 (Santa Cruz Biotechnology) in PBS solution; the secondary antibodies were Horseradish peroxidase-conjugated anti-mouse immunoglobulin rabbit polyclonal antibody (0.65 μg/ml; DAKO) and 0.25 μg/ml anti-goat immunoglobulin rabbit polyclonal antibody in 5% skimmed milk/PBS solution.

Two bands, the molecular weight of which are about 160 kD and about 140 kD, were detected in Western blot analysis of total cell extract from normal cells (WI38/SV40) (Figure 6, lane 1). The size, 160 kD, is larger than 133 kD predicted from the number of amino acids (1208 amino acids) encoded by the RecQ4 gene, suggesting the possibility

that the helicase is modified, e.g. phosphorylation, at the protein level.

RecQ4 helicase protein was undetectable in RTS-B and E cells from the patients with the antibody against the C terminus, as expected from the result of mutation analysis (Figure 6, lanes 2 and 3). Based on the above-described results, it was confirmed that the monoclonal antibody K6314 specifically recognizes the RecQ4 helicase protein. Further, in RTS-C and F, which are derived from patients with Rothmund-Thomson syndrome in whom no mutations were detected in the RecQ4 gene, RecQ4 helicase protein was detected as in normal cells (Figure 6, lanes 4 and 5). These results indicate that Western blot analysis using anti-RecQ4 helicase monoclonal antibody K6314 can be utilized to immunologically diagnose the presence of mutations in the RecQ4 gene in patients who have been diagnosed as affected with Rothmund-Thomson syndrome.

Example 7. Immunostaining of cultured cells by a method using fluorescent antibody

Intracellular localization of RecQ4 protein was analyzed by fluorescent antibody staining using the above-mentioned K6314 antibody. 0.5×10^5 cells (logarithmic growth phase) of cell line K562 derived from human chronic myelogenous leukemia were attached on a glass slide (MATSUNAMI GLASS; APS-Coated Micro Slide Glass) by using a Cytospin (TOMY SEIKO; centrifugal floating cell collector, MODEL SC-2). The cells were fixed in a solution of 3.7% formaldehyde/PBS at room temperature for 10 minutes, and then washed with PBS-T (0.05% Tween 20/PBS solution). The cell membrane permeability was enhanced in a solution of 0.1% Triton X-100/PBS at room temperature for 5 minutes. The glass slide was blocked in PBS containing 3% skimmed milk at room temperature for 1 hour and then incubation with the primary antibody was carried out in a solution containing 5 μg/ml anti-RecQ4 antibody K6314/PBS, 0.1% BSA and 0.05% NaN_3 at 4° C overnight. The glass slide was washed with PBS-T, and then incubation with the secondary antibody was performed in a solution containing 7.5 μg/ml biotin-labeled anti-mouse immunoglogulin antibody (Chemicon) at room temperature for 1 hour.

After washing with PBS-T, the glass slide was incubated in a solution of 5 μg/ml FITC-labeled streptavidin (Pharmingen) at room temperature for 1 hour and then washed with PBS-T. A solution of 2 μg/ml DAPI/50% glycerol was used to mount the sample and was counterstained for chromosomes by DAPI. Microscopic examination was carried out with an Olympas laser scanning biological microscope, FLUOVIEW system BX50.

In this observation, RecQ4 protein was detected as a very fine grain over the entire nucleoplasm (Figure 7). This result suggests that the RecQ4 protein functions in the nucleus and also that the K6314 antibody is useful in the analysis of the functions of RecQ4 helicase.

Industrial Applicability

The present invention reveals that Rothmund-Thomson syndrome is a genetic disease caused by mutations in the RecQ4 helicase gene. This finding makes it possible to conduct diagnostic tests for Rothmund-Thomson syndrome, including diagnose of a disease as Rothmund-Thomson syndrome and prenatal diagnosis for Rothmund-Thomson syndrome, and to perform treatments for Rothmund-Thomson syndrome, including gene therapy, by utilizing the RecQ4 helicase gene, primers or probes designed based on the sequence thereof, RecQ4 helicase, and antibodies thereto.

CLAIMS:

1. A genomic DNA encoding RecQ4 helicase.

2. A vector comprising the genomic DNA of claim 1.

3. A host cell containing the vector of claim 2.

4. A DNA used for the diagnosis of Rothmund-Thomson syndrome, which hybridizes to a DNA encoding the RecQ4 helicase or the expression regulatory region thereof and has a chain length of at least 15 nucleotides.

5. A therapeutic agent for Rothmund-Thomson syndrome, which contains as the effective ingredient a DNA encoding RecQ4 helicase.

6. A therapeutic agent for Rothmund-Thomson syndrome, which contains as the effective ingredient a RecQ4 helicase

7. A diagnostic agent for Rothmund-Thomson syndrome, which contains as the effective ingredient an antibody binding to RecQ4 helicase.

8. A method for the diagnosis of Rothmund-Thomson syndrome, characterized by detecting mutations in the DNA encoding RecQ4 helicase or the expression regulatory region thereof.

9. The method for the diagnosis of Rothmund-Thomson syndrome in claim 8, comprising the steps of:

(a) preparing DNA samples from patients; (b) amplifying the prepared DNA samples using the DNA of claim 4 as a primer and determining the base sequence; and

(c) comparing the determined base sequence with that of a healthy normal person.

10. The method for the diagnosis of Rothmund-Thomson syndrome in claim 8, comprising the steps of:

(a) preparing RNA samples from patients;

(b) separating the prepared RNA samples according to their size;

(c) using the DNA of claim 4 as a probe, hybridizing it to separated RNAs; and

(d) detecting the hybridized RNA and comparing the results with that of a normal, healthy person.

11. The method for the diagnosis of Rothmund-Thomson syndrome in claim 8, comprising the steps of:

(a) preparing DNA samples from patients;

(b) amplifying the prepared DNA samples using the DNA of claim 4 as a primer;

(c) dissociating the amplified DNA into single-stranded DNA;

(d) fractionating the dissociated single-stranded DNAs on a non-denaturing gel; and

(e) comparing the mobility of the fractionated single-stranded DNA on the gel with that of a healthy normal person.

12. The method for the diagnosis of Rothmund-Thomson syndrome in claim 8, comprising the steps of:

(a) preparing DNA samples from the patient;

(b) amplifying the prepared DNA samples using oligonucleotides comprising a base that forms a base pair with the mutated base specific to Rothmund-Thomson syndrome in the DNA encoding RecQ4 helicase, or the expression regulatory region thereof, as at least one of the primers; and

(c) detecting the amplified DNA fragment.

13. The method for the diagnosis of Rothmund-Thomson syndrome in claim 8, comprising the steps of:

(a) preparing DNA samples from patients;

(b) amplifying the prepared DNA samples using a pair of DNA of claim 4 which is prepared so as to flank the mutated base specific to Rothmund-Thomson syndrome as the primer;

(c) hybridizing to the amplified product a pair of oligonucleotides selected from the group of:

(i) an oligonucleotide synthesized such that the base forming a base pair with the mutated base in the amplified product corresponds to the 3'-terminus, and an oligonucleotide synthesized such that the neighboring (on the 3'side) base to said 3'-terminus corresponds to the 5'-terminus;

(ii) an oligonucleotide synthesized such that the base forming a base pair with the base of a normal healthy person which corresponds to the mutated base in the amplified product corresponds to the 3'-terminus, and an oligonucleotide synthesized such that the neighboring (on the 3'side) base to said 3'-terminus corresponds to the 5'-terminus;

(iii) an oligonucleotide synthesized such that the base

forming a base pair with the mutated base in the amplification product corresponds to the 5'-terminus, and an oligonucleotide synthesized such that the neighboring (on the 5' site) base to said 5'-terminus corresponds to the 3'-terminus; and

(iv) an oligonucleotide synthesized such that the base forming a base pair with the base of a normal healthy person which corresponds to the mutated base in the amplified product corresponds to the 5'-terminus, and an oligonucleotide synthesized such that the neighboring (on the 5' site) base to said 5'-terminus corresponds to the 3'-terminus;

(d) ligating the oligonucleotides; and

(e)detecting the ligated oligonucleotide.

14. The method for the diagnosis of Rothmund-Thomson syndrome in claim 8, comprising the steps of:

(a) preparing protein samples from patients;

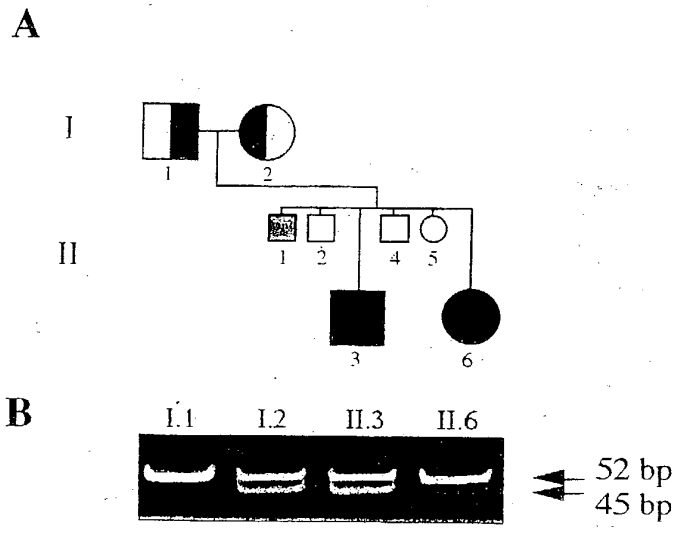
(b) contacting an antibody against RecQ4 helicase with the prepared protein sample; and

(c) detecting proteins binding to said antibody.

ABSTRACT

The RecQ4 helicase gene, belonging to the RecQ helicase gene family, is revealed herein to be the causative gene of Rothmund-Thomson syndrome. The present inventors found out that it is possible to diagnose Rothmund-Thomson syndrome by detecting mutation of this gene. Further, they uncovered that it is possible to treat patients of Rothmund-Thomson syndrome by utilizing normal RecQ4 helicase gene or proteins thereof.

Figure 1



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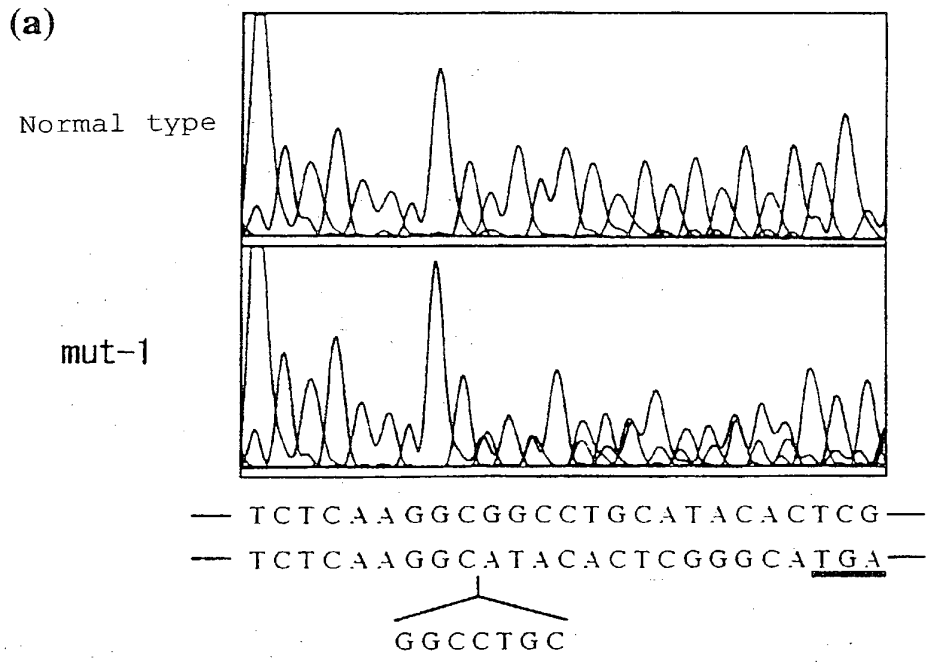

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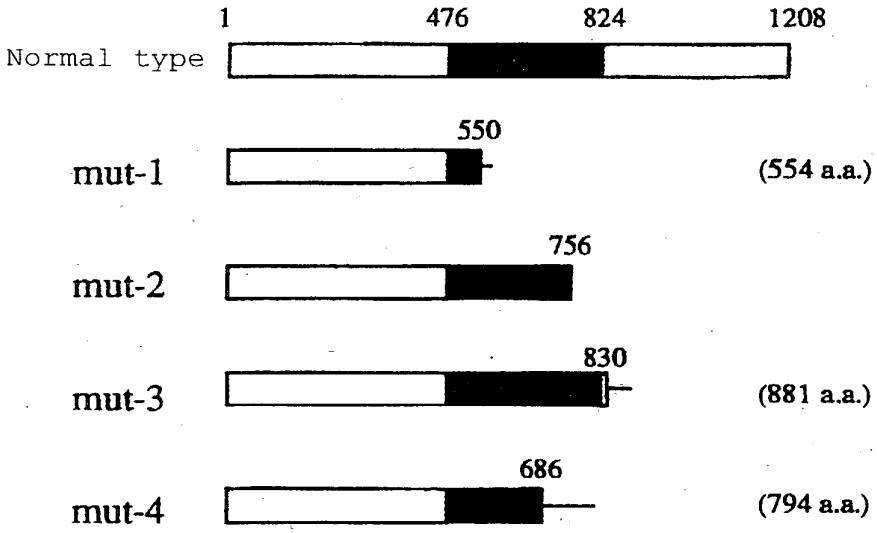
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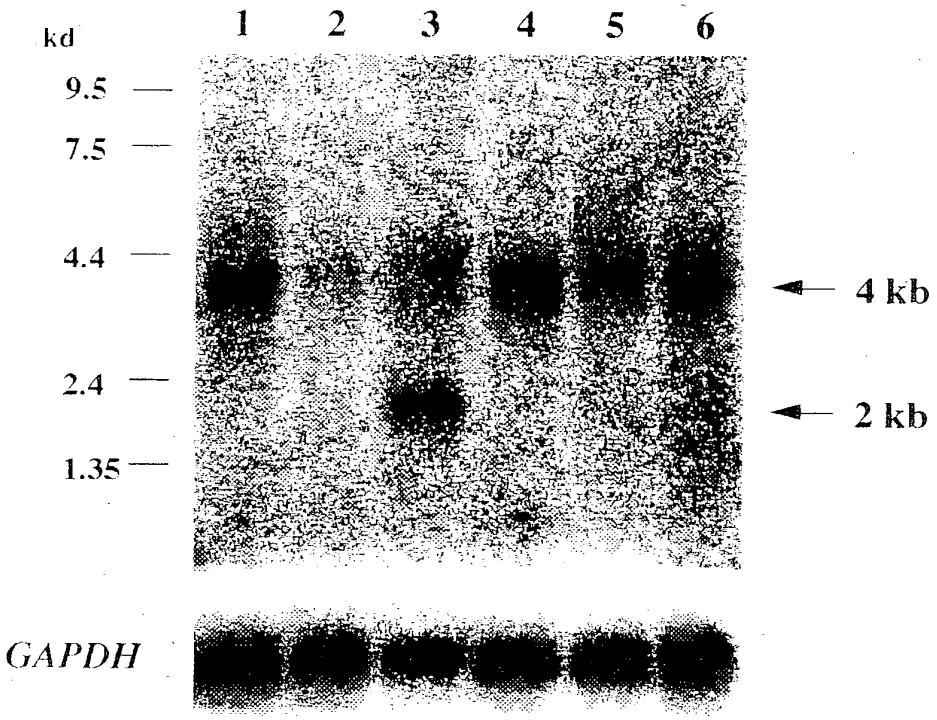

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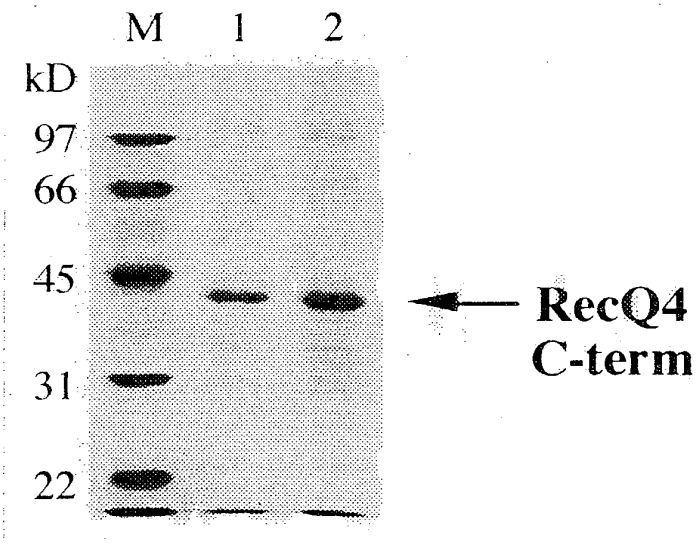

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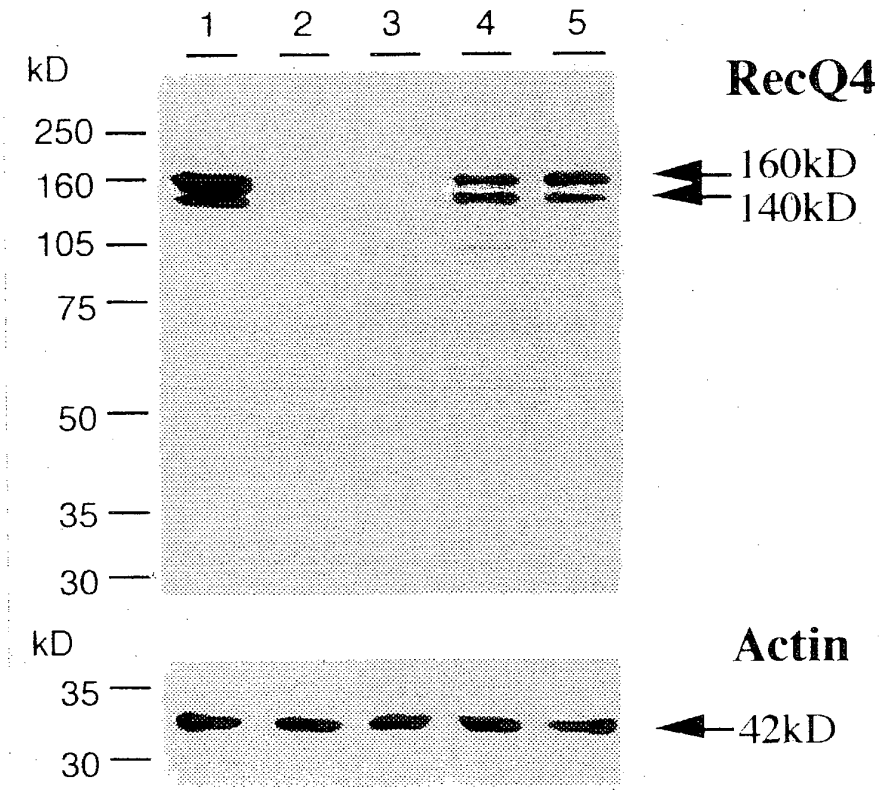

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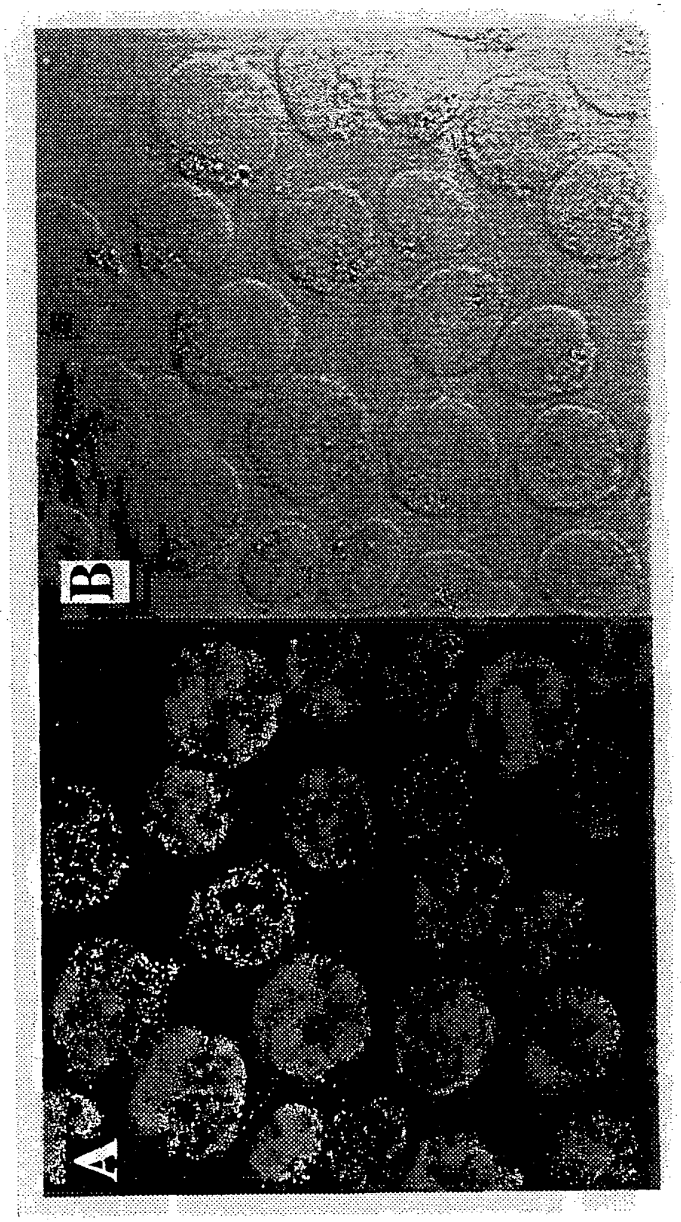

Banner & Witcoff Ref. No. 04276.00003
Client Ref. No. A1-003PCT-US

JOINT DECLARATION FOR PATENT APPLICATION

As the below named inventors, we hereby declare that:

Our residence, post office address and citizenship are as stated below next to our names;

We believe we are the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled GENE CAUSATIVE OF ROTHMUND-THOMSON SYNDROME AND ITS GENE PRODUCT, the specification of which

- ☐ is attached hereto.
- ☐ was filed on _____as Application Serial Number _____and was amended on _____ (if applicable).
- ☒ was filed under the Patent Cooperation Treaty (PCT) and accorded International Application No. PCT/JP00/00233, filed January 19, 2000, and amended on _____ (if any).

We hereby state that we have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We hereby acknowledge the duty to disclose information which is material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

Prior Foreign Application(s)

We hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application(s) for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Country	Application No.	Date of Filing (day, month, year)	Date of Issue (day, month, year)	Priority Claimed Under 35 U.S.C. §119
Japan	11/11218	19 January 1999		Yes
PCT	PCT/JP00/00233	19 January 2000		Yes

Prior United States Provisional Application(s)

We hereby claim priority benefits under Title 35, United States Code, §119(e)(1) of any U.S. provisional application listed below:

U.S. Provisional Application No.	Date of Filing (day, month, year)	Priority Claimed Under 35 U.S.C. §119(e)(1)

Prior United States Application(s)

We hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, we acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Date of Filing (Day, Month, Year)	Status X Patented, Pending, Abandoned

Banner & Witcoff Ref. No. 04276.00003
Client Ref. No. A1-003PCT-US

Power of Attorney

And we hereby appoint, both jointly and severally, as our attorneys with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith the following at attorneys and agents, their registration numbers being listed after their names:

ALTHERR, Robert F.	31,810	HONG, Patricia E.	34,373	NELSON, Jon O.	24,566
BANNER, Donald W.	17,037	HOSCHEIT, Dale H.	19,090	NIEGOWSKI, James A.	28,331
BANNER, Mark T.	29,888	HYMEL, Lin J.	45,414	PATEL, Binal J.	42,065
BANNER, Pamela I.	33,644	IWANICKI, John P.	34,628	PATHAK, Ajay S.	38,266
BECKER, Matthew P.	45,824	JACKSON, Thomas H.	29,808	PETERSON, Thomas L.	30,969
BECKETT, William W.	18,262	KAGAN, Sarah A.	32,141	POTENZA, Joseph M.	28,175
BERGHAMMER, Joseph J.	46,057	KATZ, Robert S.	36,402	PRATT, Thomas K.	37,210
BODNER, Jordan	42,338	KLEIN, William J.	43,719	RENK, Christopher J.	33,761
BUROW, Scott A.	42,373	KRAUSE, Joseph P.	32,578	RESIS, Robert H.	32,168
CALLAHAN, James V.	20,095	LINEK, Ernest V.	29,822	RIVARD, Paul M.	43,446
CHANG, Steve S	42,402	MALONE, Dale A.	32,155	ROBINSON, Douglas W.	32,751
COHAN, Gregory J.	40,959	MANNAVA, Ashok K.	45,301	SCHAD, Steven P.	32,550
COOPERMAN, Marc S.	34,143	MAPLE, Marie-Claire B.	37,588	SHIFLEY, Charles W.	28,042
CURTIN, Joseph P.	34,571	MAY, Steven A.	44,912	SHULL, Jason	47,085
DAVID, Michael	44,642	McDERMOTT, Peter D.	29,411	SKERPON, Joseph M.	29,864
DeMOOR, Laura J.	39,654	McKEE, Christopher L.	32,384	STOCKLEY, D. J.	34,257
EVANS, Thomas L.	35,805	McKIE, Edward F.	17,335	VAN ES, J. Pieter	37,746
FEDOROCHKO, Gary D.	35,509	MEDLOCK, Nina L.	29,673	WITCOFF, Sheldon W.	17,399
FERGUSON, Catherine A.	40,877	MEECE, Timothy C.	38,553	WOLFFE, Franklin D.	19,724
FICKLER, Debra A.	46,699	MEEKER, Frederic M.	35,282	WOLFFE, Susan A.	33,568
FISHER, William J.	32,133	MILLER, Charles L.	43,805	WRIGHT, Bradley C.	38,061
GLEMBOCKI, Christopher R.	38,800	MITRIUS, Janice V.	43,808		
HANLON, Brian E.	40,449	MORENO, Christopher P.	38,566		
HEMMENDINGER, Lisa M.	42,653	MOTTLEY, Darrell G.	42,912		

All correspondence and telephone communications should be addressed to:

Banner & Witcoff, Ltd.
Customer Number: 22907 (WDC)

Banner & Witcoff Ref. No. 04276.00003
Client Ref. No. A1-003PCT-US

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1-00 Signature Saori Kitao Date August 3, 2001
Full Name of First Inventor KITAO (Family Name) Saori (First Given Name) (Second Given Name)
Residence Kanagawa, JAPAN JPX Citizenship Japan
Post Office Address c/o GENECARE RESEARCH INSTITUTE CO., LTD.,
200, Kajiwara, Kamakura-shi, Kanagawa 247-0063, JAPAN

2-00 Signature Akira Shimamoto Date August 3, 2001
Full Name of Second Inventor SHIMAMOTO (Family Name) Akira (First Given Name) (Second Given Name)
Residence Kanagawa, JAPAN JPX Citizenship Japan
Post Office Address c/o GENECARE RESEARCH INSTITUTE CO., LTD.,
200, Kajiwara, Kamakura-shi, Kanagawa 247-0063, JAPAN

3-00 Signature Yasuhiro Furuichi Date August 10, 2001
Full Name of Third Inventor FURUICHI (Family Name) Yasuhiro (First Given Name) (Second Given Name)
Residence Kanagawa, JAPAN JPX Citizenship Japan
Post Office Address c/o GENECARE RESEARCH INSTITUTE CO., LTD.,
200, Kajiwara, Kamakura-shi, Kanagawa 247-0063, JAPAN

SEQUENCE LISTING

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and its gene product

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Val Lys Arg Leu Val Gln Arg Val Phe Pro Ala Cys Thr Cys Thr Cys
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Val Pro Lys Tyr Pro Pro Gln Glu Ala Glu Gln Leu Ser His Gln Ala
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Arg Pro Asp Leu Gly Ser Glu Glu Ser Gln Leu Leu Ile Pro Gly Glu
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Gln Arg Glu Ser Val Leu Gln Lys Ile Arg Ala Ala Gln Val His Val
565 570 575

Leu Met Leu Thr Pro Glu Ala Leu Val Gly Ala Gly Gly Leu Pro Pro
580 585 590

Ala Ala Gln Leu Pro Pro Val Ala Phe Ala Cys Ile Asp Glu Ala His
595 600 605

Cys Leu Ser Gln Trp Ser His Asn Phe Arg Pro Cys Tyr Leu Arg Val
610 615 620

Cys Lys Val Leu Arg Glu Arg Met Gly Val His Cys Phe Leu Gly Leu
625 630 635 640

Thr Ala Thr Ala Thr Arg Arg Thr Ala Ser Asp Val Ala Gln His Leu
645 650 655

Ala Val Ala Glu Glu Pro Asp Leu His Gly Pro Ala Pro Val Pro Thr
660 665 670

Asn Leu His Leu Ser Val Ser Met Asp Arg Asp Thr Asp Gln Ala Leu
675 680 685

Leu Thr Leu Leu Gln Gly Lys Arg Phe Gln Asn Leu Asp Ser Ile Ile
690 695 700

Ile Tyr Cys Asn Arg Arg Glu Asp Thr Glu Arg Ile Ala Ala Leu Leu
705 710 715 720

Arg Thr Cys Leu His Ala Ala Trp Val Pro Gly Ser Gly Gly Arg Ala
725 730 735

Pro Lys Thr Thr Ala Glu Ala Tyr His Ala Gly Met Cys Ser Arg Glu
740 745 750

Arg Arg Arg Val Gln Arg Ala Phe Met Gln Gly Gln Leu Arg Val Val

755 760 765

Val Ala Thr Val Ala Phe Gly Met Gly Leu Asp Arg Pro Asp Val Arg
770 775 780

Ala Val Leu His Leu Gly Leu Pro Pro Ser Phe Glu Ser Tyr Val Gln
785 790 795 800

Ala Val Gly Arg Ala Gly Arg Asp Gly Gln Pro Ala His Cys His Leu
805 810 815

Phe Leu Gln Pro Gln Gly Glu Asp Leu Arg Glu Leu Arg Arg His Val
820 825 830

His Ala Asp Ser Thr Asp Phe Leu Ala Val Lys Arg Leu Val Gln Arg
835 840 845

Val Phe Pro Ala Cys Thr Cys Thr Cys Thr Arg Pro Pro Ser Glu Gln
850 855 860

Glu Gly Ala Val Gly Gly Glu Arg Pro Val Pro Lys Tyr Pro Pro Gln
865 870 875 880

Glu Ala Glu Gln Leu Ser His Gln Ala Ala Pro Gly Pro Arg Arg Val
885 890 895

Cys Met Gly His Glu Arg Ala Leu Pro Ile Gln Leu Thr Val Gln Ala
900 905 910

Leu Asp Met Pro Glu Glu Ala Ile Glu Thr Leu Leu Cys Tyr Leu Glu
915 920 925

Leu His Pro His His Trp Leu Glu Leu Leu Ala Thr Thr Tyr Thr His
930 935 940

Cys Arg Leu Asn Cys Pro Gly Gly Pro Ala Gln Leu Gln Ala Leu Ala
945 950 955 960

His Arg Cys Pro Pro Leu Ala Val Cys Leu Ala Gln Gln Leu Pro Glu
965 970 975

Asp Pro Gly Gln Gly Ser Ser Ser Val Glu Phe Asp Met Val Lys Leu
980 985 990

Val Asp Ser Met Gly Trp Glu Leu Ala Ser Val Arg Arg Ala Leu Cys
995 1000 1005

Gln Leu Gln Trp Asp His Glu Pro Arg Thr Gly Val Arg Arg Gly Thr
1010 1015 1020

Gly Val Leu Val Glu Phe Ser Glu Leu Ala Phe His Leu Arg Ser Pro
1025 1030 1035 1040

Gly Asp Leu Thr Ala Glu Glu Lys Asp Gln Ile Cys Asp Phe Leu Tyr
1045 1050 1055

Gly Arg Val Gln Ala Arg Glu Arg Gln Ala Leu Ala Arg Leu Arg Arg
1060 1065 1070

Thr Phe Gln Ala Phe His Ser Val Ala Phe Pro Ser Cys Gly Pro Cys
1075 1080 1085

Leu Glu Gln Gln Asp Glu Glu Arg Ser Thr Arg Leu Lys Asp Leu Leu
1090 1095 1100

Gly Arg Tyr Phe Glu Glu Glu Glu Gly Gln Glu Pro Gly Gly Met Glu
1105 1110 1115 1120

Asp Ala Gln Gly Pro Glu Pro Gly Gln Ala Arg Leu Gln Asp Trp Glu
1125 1130 1135

Asp Gln Val Arg Cys Asp Ile Arg Gln Phe Leu Ser Leu Arg Pro Glu
1140 1145 1150

Glu Lys Phe Ser Ser Arg Ala Val Ala Arg Ile Phe His Gly Ile Gly
1155 1160 1165

Ser Pro Cys Tyr Pro Ala Gln Val Tyr Gly Gln Asp Arg Arg Phe Trp

1170 1175 1180

Arg Lys Tyr Leu His Leu Ser Phe His Ala Leu Val Gly Leu Ala Thr
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Glu Glu Leu Leu Gln Val Ala Arg
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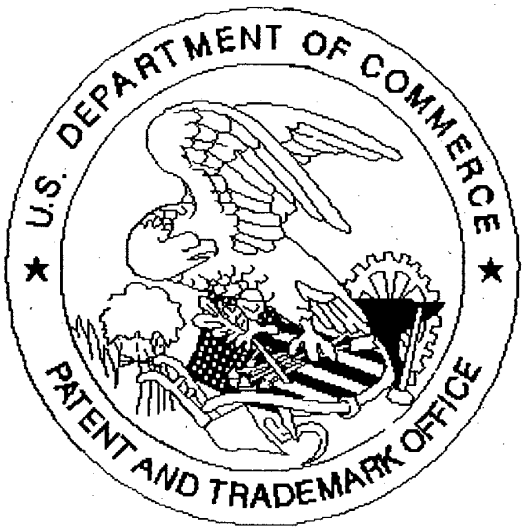

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☐ Page(s)_____ of _____ were not present for scanning. (Document title)

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